



(19)

Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 1 327 688 A1

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:

16.07.2003 Bulletin 2003/29

(51) Int Cl.7: C12N 15/86, C07K 14/47

(21) Application number: 02075108.7

(22) Date of filing: 14.01.2002

(84) Designated Contracting States:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE TR

Designated Extension States:

AL LT LV MK RO SI

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(54) **Adenoviruses with enhanced lytic potency**

(57) Described is a recombinant virus, being capable to replicate in target cells, and having lytic capacity in the said cells, the virus comprising in the genome thereof, the coding sequence of at least one protein functional in restoring the p53 apoptosis pathway in the said target cells, operably linked to one or more expres-

sion control sequences, functional in the said target cells, as well as the use thereof in the preparation of a medicament, in particular for suppressing uncontrolled cell growth.

Description

Field of the invention

[0001] The present invention relates to the fields of genetic modification, biotechnology and medicine. In particular, the invention provides recombinant adenoviruses with an enhanced potency to lyse cells in which they replicate. As a result, the adenoviruses of the invention replicate faster within a population of cells. The invention thus provides efficient production of recombinant adenoviruses and of proteins encoded by said recombinant adenoviruses, as well as efficient means to eradicate certain populations of cells. The invention finds useful applications in the areas of viral vector production, recombinant protein production, vaccine production, and medical treatments based on removal of certain cells from a body, such as e.g. cancer cells.

Background of the invention

[0002] Recombinant adenoviruses are generated from the genome of adenoviruses through genetic engineering. This genetic engineering often involves insertion of heterologous DNA, including but not limited to DNA encoding a therapeutic product, into the adenovirus genome. It is to be understood, however, that the term recombinant adenovirus is also meant to include adenovirus from which parts of the virus genome have been removed without insertion of heterologous DNA. Another example of a recombinant adenovirus is a chimeric adenovirus containing parts of the genomes of different adenoviruses. When said parts of the adenovirus genome that are removed include parts that are essential for at least one step of the adenovirus infectious life cycle, the recombinant adenovirus is replication deficient, which means that said recombinant adenovirus alone is incapable of completing the adenovirus infectious life cycle. This type of recombinant adenovirus can, therefore, only replicate in a cell when the functions encoded by said removed parts of the genome are provided in said cell by other means. In a second type of recombinant adenovirus said parts that are essential for at least one step of the adenovirus infectious life cycle are also removed, but the essential functions of said parts are complemented by inserting functional expression cassettes for heterologous proteins that provide said essential functions in the recombinant adenovirus genome. This type of recombinant adenovirus is referred to herein as a heterologously trans-complemented adenovirus. A third type of recombinant adenovirus is the so-called conditionally replicating adenovirus (CRAd). In CRAds said parts of the adenovirus genome that are removed include parts that are essential for at least one step of the adenovirus infectious life cycle under certain conditions but not under certain other conditions. Said other conditions could, e.g., mean the conditions that exist in a particular type of cell, in a particular

stage of the cell cycle, in a particular developmental stage of the cell, in the presence of particular compounds, and the like. In a fourth type of recombinant adenovirus no parts of the adenovirus genome have been removed or said parts of the adenovirus genome that are removed do not include parts that are essential for at least one step of the adenovirus infectious life cycle. This type of recombinant adenovirus is, therefore, a replication competent adenovirus that has a capacity to replicate in cells like the parental unmodified adenovirus does. In general, the replication of recombinant adenoviruses is restricted to cells of a particular animal species or group of animal species. E.g., recombinant adenoviruses derived from human adenoviruses can only transverse a complete life cycle in human cells, with very inefficient replication occurring at high dose in cells of some other species.

[0003] The production of recombinant adenoviruses usually starts with genetic engineering of at least a part of the adenovirus genome by standard molecular biology techniques known in the art. In some cases, a full-length recombinant adenovirus construct comprising all elements required for replication in at least a certain type of cell is made, in other cases the recombinant adenovirus genome is separated over two or more constructs that share sequence homology. Next, the adenovirus genome (comprised by one or more constructs) is introduced into cells that allow replication of said recombinant adenovirus by DNA transfer methods known in the art, including but not restricted to calcium phosphate precipitation, DEAE-dextran mediated transfection, lipofectamine mediated transfection, electroporation, and the like. Procedures using an adenovirus genome that is separated over more than one construct rely on homologous recombination between the parts of the adenovirus genome that are shared by the constructs to occur in said cells to constitute a complete recombinant adenovirus genome.

[0004] After the recombinant adenovirus has started to replicate in cells into which the recombinant adenovirus genome has been introduced, said recombinant adenovirus can spread to other cells in the culture. The recombinant adenovirus can also be isolated from the culture medium or from lysates of the cells in which said recombinant adenovirus is replicating. The isolated recombinant adenovirus vector can then be used to re-infect new cells to further propagate and expand said recombinant adenovirus. In addition, said recombinant adenovirus can be administered to an animal body to infect cells *in vivo*. This administration can be done via several routes, including but not limited to direct injection into a tissue, oral administration, injection into the blood circulation, inhalation, injection into a body cavity, and application to the surface of a certain body area. Following infection of said cells *in vivo*, the recombinant adenovirus can replicate and spread to other cells *in vivo*, provided that the infected cells support replication of said recombinant adenovirus.

[0005] Replication deficient adenoviruses will not replicate in most cells in an animal body, except for special cell types that complement for the parts of the adenovirus genome that are removed and that are essential for the adenovirus infectious life cycle. Such special cell types include certain (growth factor-stimulated) cancer cells that exhibit what is generally referred to as "E1A-like activity" (Spergel and Chen-Kiang, Proc. Natl. Acad. Sci. USA 88(1991):6472-6476; Rancourt et al, Clin. Cancer Res. 5(1999):43-50; Steinwaerder et al, Hum. Gene Ther. 11(2000):1933-1948). Replication competent adenoviruses will replicate in many different cells in an animal body, provided that they are derived from adenoviruses with the correct species tropism and that said cells express surface receptors for said adenoviruses. CRAds will only replicate in cells in which the particular conditions exist that are required for replication of the CRAd. CRAds are designed to meet the specific requirements for replication in a chosen type of cell and not in other types of cells.

[0006] The adenovirus replication process constitutes the following steps: (1) binding of the adenovirus particle to the surface of the host cell via receptor molecules, (2) internalization of the virus particle by endocytosis, (3) escape from the endosome into the cytoplasm and transport towards the cell nucleus, during which the virus particle is partially broken down, (4) import of the adenovirus DNA genome into the cell nucleus, (5) expression of adenovirus proteins encoded by the early regions in the adenovirus genome, (6) replication of the adenovirus genome, (7) expression of adenovirus proteins encoded by the late regions in the adenovirus genome, (8) assembly of progeny adenovirus particles and inclusion of progeny adenovirus genomes into these particles, (9) induction of cell death, leading to (10) release of adenovirus progeny from the cell.

[0007] Important natural target cells for adenoviruses are non-dividing epithelial cells. These cells lack active machinery for the synthesis of DNA. Therefore, in order to replicate their DNA genome in these cells, adenoviruses induce the cellular DNA synthetic machinery. Adenovirus proteins encoded by the early region 1A (E1A) are potent inducers of DNA synthesis, cell growth and transformation, which they bring about through the formation of complexes with cellular proteins involved in cell cycle control. These effects of E1A cause cytotoxicity and induction of programmed cell death or apoptosis. In different cell lines, p53 dependent as well as p53 independent apoptosis has been documented after adenovirus infection (Teodoro and Branton, J. Virol. 71 (1997):1739-1746; and references therein). Two proteins encoded by the early region 1B (E1B), E1B-19kDa and E1B-55kDa, and the early region 4 orf6 protein (E4orf6) suppress the cytotoxicity and apoptosis induced by E1A. The E1B-55kDa and E4orf6 proteins co-operate to suppress apoptosis at least in part by forming a complex with p53 and inhibiting p53-mediated transactivation as well as promoting p53 degradation. The

E1B-19kDa protein interacts with proteins of the bcl-2 family to inhibit the caspase-9 dependent apoptosis pathway. The suppression of apoptosis prevents premature cell death, thereby allowing the adenovirus to complete its life cycle in the cell. In contrast, at late stages of infection cell death and lysis promotes release of the virus progeny from the cell. An important mechanism used by adenovirus to accomplish this is through induction of apoptosis. Adenovirus proteins that were shown to be involved in late apoptosis induction or efficient cell lysis at late stages of infection by a currently unresolved mechanism include the E4orf4 protein (Shtrichman and Kleinberger, J. Virol. 72(1998):2975-2982; Marcellus et al, J. Virol. 72(1998):7144-7153) and the E3-11.6kDa nuclear membrane glycoprotein, also termed adenovirus death protein (ADP) (Tollefson et al, J. Virol. 70 (1996):2296-2306).

[0008] Cancer cells and cell lines are the result of neoplastic transformation. The genetic events underlying neoplastic transformation include activation of proto-oncogenes and inactivation of tumor-suppressor genes. A major player in this respect is the gene encoding the tumor-suppressor protein p53. The loss of normal function of p53 is associated with resistance to apoptosis, cell transformation *in vitro* and development of neoplasms *in vivo*. In fact, in approximately 50% of human cancers the gene encoding p53 is non-functional through deletion or mutation (Levine et al, Nature 351(1991): 453-456; Hollstein et al, Science 253(1991):49-53; Chang et al, J. Clin. Oncol. 13(1995):1009-1022). Also in cancer cells that do express wild-type p53 protein, apoptosis is hampered. At least in some cases and perhaps in all cases, this is the result of functional inactivation of p53 in these cells. E.g., loss of the tumor-suppressor protein p14ARF or overexpression of MDM2 protein can lead to functional inactivation of p53 by binding to the MDM2 protein and subsequent degradation (Landers et al., Oncogene 9(1994):2745-2750; Florenes et al., J. Natl. Cancer Inst. 86(1994): 1297-1302; Blaydes et al., Oncogene 14(1997): 1859-1868; Stott et al, EMBO J. 17(1998):5001-5014; Schmitt et al, Genes Dev. 19(1999):2670-2677). Another example is functional inactivation of p53 as a result of the antagonizing binding of human papilloma virus (HPV) E6 protein in cervical carcinomas (Scheffner et al., Cell 63(1990):1129-1136) or of herpesvirus-8 latency-associated nuclear antigen in Kaposi's sarcoma (Friberg et al., Nature 402(1999):889-894). Thus, in many if not all cancers *in vivo* and cancer-derived or immortalized cell lines *in vitro* apoptosis is hampered as a result of one or more lesions in the p53-dependent pathway.

[0009] Loss of p53 function has also been documented in other diseases involving inappropriate cell survival, such as for example rheumatoid arthritis (Firestein et al., J. Clin. Invest. 96(1995):1631-1638; Firestein et al., Am. J. Pathol. 149(1996):2143-2151; Firestein et al., Proc. Natl. Acad. Sci. USA 94(1997):10895-10900) and

vascular smooth muscle cell hyperplasia (Speir et al., Science 265(1994):391-394; Kovacs et al., Am J. Pathol. 149(1996):1531-1539).

[0010] Interestingly, it has been observed that replicating adenoviruses kill cell lines that express functional p53 more rapidly than cell lines that are deficient in p53 (Hall et al., Nature Med. 4(1998):1068-1072; Goodrum and Ornelles, J. Virol. 72(1998):9479-9490; Dix et al., Cancer Res. 60(2000):2666-2672). In addition, the formation of a complex between p53 and the E1B-55kDa protein was found to be essential for this rapid induction of cell death by adenovirus. It was concluded that adenoviruses exploit multiple cell death processes, of which a p53 and E1B-55kDa protein dependent pathway is the most rapid one (Dix et al., Cancer Res. 60(2000): 2666-2672).

[0011] The p53 protein is the central coordinator of damage-induced cell-cycle checkpoint control. In a perturbed cell, p53 can simultaneously induce growth arrest and apoptosis. p53 exerts these effects by functioning as a specific transcription factor that controls the expression of a large panel of genes involved in growth control, DNA repair, cell-cycle arrest, apoptosis promotion, redox regulation, nitric oxide production, and protein degradation (Polyak et al., Nature 389(1997): 237-238; El-Deiry, Sem. Cancer. Biol. 8(1998):345-357; Yu et al., Proc. Natl. Acad. Sci. USA 96(1999): 14517-14522; Hupp et al., Biochem. J. 352(2000):1-17; and references therein). The induction of apoptosis by p53 is mediated at least in part by activation of pro-apoptotic death genes of the *bcl-2* family, such as *bax*, *bak*, and *bcl-x_s* (Miyashita and Reed, Cell 80(1995):293-299; Han et al., Genes Dev. 10(1996):461-477). The immediate effector proteins of p53 as well as p53 itself target mitochondria, thereby releasing cytochrome c into the cytosol to activate the caspase cascade via the initiator caspase-9/Apaf-1 complex (Juergensmeier et al., Proc. Natl. Acad. Sci. USA 95(1998):4997-5002; Fearnhead et al., Proc. Natl. Acad. Sci. USA 95(1998): 13664-13669; Soengas et al., Science 284(1999): 156-159; Marchenko et al., J. Biol. Chem. 275(2000): 16202-16212). There is evidence from mutation analysis that the transcription activation functions of p53 responsible for growth arrest and apoptosis can be dissected. For example, the p53 Q22/S23-mutant protein has abrogated growth arrest function but only attenuated apoptosis induction capacity (Venot et al., Oncogene 18(1999):2405-2410). Furthermore, several p53 homologues have been identified, including p73 and p63, which share part of the functions with p53 (Kaghad et al., Cell 90(1997):809-819; Yang et al., Mol. Cell 2 (1998):305-316). In the presence of the adenovirus E1B-19kDa protein, which binds to and inactivates pro-apoptotic death genes of the *bcl-2* family, the p53-dependent growth arrest pathway becomes apparent. Otherwise, apoptosis is dominant over growth arrest (Han et al., Genes Dev. 10(1996):461-477).

[0012] Recombinant adenoviruses are finding in-

creasing utility for the treatment of cancer and other diseases involving inappropriate cell survival. In particular, CRAds have been developed to selectively replicate in and kill cancer cells. Such cancer-specific CRAds represent a novel and very promising class of anticancer agents (reviewed by: Heise and Kirn, J. Clin. Invest. 105 (2000):847-851; Alemany et al., Nat. Biotech. 18(2000): 723-727; Gomez-Navarro and Curiel, Lancet Oncol. 1 (2000):148-158). The tumor-selective replication of this

5 type of CRAds is achieved through either of two alternative strategies. In the first strategy, the expression of an essential early adenovirus gene is controlled by a tumor-specific promoter (Rodriguez et al., Cancer Res. 57 (1997):2559-2563; Hallenbeck et al., Hum. Gene Ther. 10(1999):1721-1733). The second strategy involves the 10 introduction of mutations in viral genes to abrogate the interaction of the encoded proteins with cellular proteins, necessary to complete the viral life cycle in normal cells, but not in tumor cells (Bischoff et al., Science 274 15 (1996):373-376; Fueyo et al., Oncogene 19(2000):2-12; Heise et al., Clin. Cancer Res. 6(2000):4908-4914). During their replication in tumor cells CRAds destroy 20 these cells by inducing lysis, a process that is further referred to as "oncolysis". The release of viral progeny 25 from lysed tumor cells offers the potential to amplify CRAds *in situ* and to achieve lateral spread to neighboring cells in a solid tumor, thus expanding the oncolytic effect. The restriction of CRAd replication to tumor or 30 hyperproliferative cells dictates the safety of the agent, by preventing lysis of normal tissue cells. Currently, CRAd-based cancer treatments are already being evaluated in clinical trials (e.g., Nemunaitis et al., Cancer Res. 60(2000):6359-6366; Khuri et al., Nature Med. 6 (2000):879-885; Habib et al., Hum. Gene Ther. 12 35 (2001):219-226).

[0013] However, despite very encouraging results from *in vitro* and animal studies, the anti-cancer efficacy of CRAds as a single agent in humans has been limited (Kirn et al., Nature Med. 4(1998):1341-1342; Ganly et 40 al., Clin. Cancer Res. 6(2000):798-806; Nemunaitis et al., Cancer Res. 60(2000):6359-6366; Mulvihill et al., Gene Therapy 8(2001):308-315). Thus, there is a clear 45 need in the field of cancer treatment to increase the potency of recombinant adenoviruses as oncolytic agents.

This could be achieved by enhancing their replication and lysis capacities.

[0014] Several approaches aimed at improving the replication and lysis capacities of recombinant adenoviruses, or at preventing loss of these functions from the 50 wild-type adenovirus have been taken. It has been shown that it is better to retain the adenovirus E3 region in a recombinant adenovirus (Yu et al., Cancer Res. 60 (2000):4200-4203) or, in case most of the E3 region is deleted, to at least retain the gene encoding the 55 E3-11.6kDa protein (Tollefson et al., J. Virol. 70(1996): 2296-2306; Doronin et al., J. Virol. 74(2000): 6147-6155). In addition, replication and cell lysis of recombinant adenoviruses have been improved by incor-

poration of cytotoxic genes (Zhang et al, Proc. Natl. Acad. Sci. USA 93(1996):4513-4518; Freytag et al, Hum. Gene Ther. 9(1998):1323-1333; Wildner et al, Gene Ther. 6(1999):57-62). It was also shown that recombinant adenoviruses are more potent in killing cancer cells when they contain the complete E1A region, but lack the E1B-19kDa protein (Martin Duque et al, Cancer Gene Ther. 6(1999):554-563; Sauthoff et al, Hum. Gene Ther. 11(2000):379-388). Finally, the release of a recombinant adenovirus with deleted E1 and E3 regions from HeLa cervix cancer cells was enhanced by inducing apoptosis in these cells (Mi et al, Hum. Gene Ther. 12(2001):1343-1352). In the latter case, it was crucial that apoptosis was induced after progeny virus assembly in the cell had been completed. Premature apoptosis induction during viral DNA replication compromised virus production.

Brief description of the invention

[0015] In many instances it is preferred that a recombinant adenovirus undergoes a rapid life cycle in a host cell. When a recombinant adenovirus is produced or when a recombinant adenovirus is used as a vector to produce a protein in cells, a rapid adenovirus life cycle speeds up the production process. When a recombinant adenovirus is used as a means to kill a population of cells, a rapid life cycle will add to the efficacy of the process. A rapid life cycle is of particular importance for the use of a recombinant adenovirus *in vivo*. Adenoviruses induce potent immune responses in the body of animals that inactivate said adenoviruses. This limits the duration of *in vivo* replication of an administered recombinant adenovirus. A faster life cycle will thus allow more cycles of progeny virus production within the time-span between administration and inactivation of the recombinant adenovirus. A situation where a rapid life cycle of a recombinant adenovirus is of particular importance *in vivo* is in the context of the treatment of a disease involving inappropriate cell survival. A paradigm example of such a disease is cancer. The anticancer potency of a recombinant adenovirus that is administered to a tumor *in vivo* depends on (1) the efficiency at which the virus disseminates throughout the tumor by producing progeny that can infect neighboring tumor cells, and (2) the efficiency at which the virus kills tumor cells via replication and lysis of the said cells. Thus, a rapid life cycle will result in a faster oncolysis, more cycles of new virus production per time, infection of more tumor cells in time, and, consequently, more effective tumor destruction.

[0016] Therefore, an important objective of the present invention is to provide recombinant adenoviruses that have a short replication time in a host cell. Said replication time is understood to mean the time between entry of the recombinant adenovirus into the cell and the release of progeny of said recombinant adenovirus from the cell.

[0017] It is a second objective of the present invention

to provide recombinant adenoviruses that have a fast lytic capacity. A fast lytic capacity is understood to mean a short time required to lyse a host cell after entry of the recombinant adenovirus into said host cell.

5 **[0018]** In preferred embodiments of the invention, said host cell in which said recombinant adenoviruses have a short replication time and/or a fast lytic capacity is a cell with insufficient capacity to respond to a loss of cell-cycle checkpoint control, where it is further preferred that said cell is a human cell. Non-limiting examples of host cells according to the invention are cancer or tumor cells, arthritic cells, hyperproliferative vascular smooth muscle cells and cells infected with a DNA virus other than said recombinant adenovirus.

10 **[0019]** In one variation of the invention, said host cell is a cell that is being cultured *in vitro*. In another variation of the invention, said host cell is a cell in an animal body, where it is preferred that said animal body is a human body.

15 **[0020]** In a preferred embodiment of the invention, said fast lytic capacity is the result of induction of cell death, where it is preferred that said cell death involves the p53-dependent apoptosis pathway.

20 **[0021]** The concept of the present invention is based on the following line of reasoning:

25 (1) To increase the rate of replication of a recombinant adenovirus in a cell at least one step of the replication process needs to be augmented.

30 (2) The step during the adenovirus life cycle that most critically determines the rate of virus replication is the step that requires the most time.

35 (3) For the type of adenovirus that is most widely used to generate recombinant adenoviruses, i.e., human adenovirus serotype 5, the steps 1-8 of the life cycle as described in the background of the invention are completed within approximately 2 days after cell entry. In contrast, the induction of lysis at late stages of infection occurs with various rates, depending on the host cell type, and may take as long as one week.

40 (4) Therefore, augmenting the lytic capacity of a recombinant adenovirus should have a major impact on the length of the recombinant adenovirus life cycle.

45 (5) The speed at which recombinant adenoviruses induce cell lysis appears related to the p53 status of the cell, where p53 deficiency correlates with delayed lysis.

50 (6) Many if not all hyperproliferative cells including cancer cells and immortalized cell lines carry one or more lesions in the p53-dependent apoptosis pathway.

55 (7) Hence, it should be possible to increase the speed at which recombinant adenoviruses induce cell lysis and complete their life cycle in a host cell by restoring a functional p53-dependent apoptosis pathway in the host cell during recombinant aden-

ovirus replication.

[0022] The invention thus provides recombinant adenoviruses capable of replicating in a host cell and expressing a functional protein of the p53-dependent apoptosis pathway. Non-limiting examples of said functional protein are p53 protein, BAX protein, and family members thereof. In a preferred embodiment, said recombinant adenovirus is a replication competent adenovirus or a conditionally replicating adenovirus. It is furthermore preferred that said protein is a mammalian protein, for example a human protein.

[0023] The term "functional protein" means that said protein comprises at least one activity of the natural counterpart (i.e., the wild-type) of said protein, where "activity" means at least in nature. It is also preferred that the activity at least equals that of the natural counterpart in amount, but is preferably even higher. Thus, "functional protein" includes the wild-type protein and all its natural or synthetic derivatives that share at least one activity with said wild-type protein. The term "protein" is meant to include peptides or functional fragments of proteins or peptides.

[0024] The invention is furthermore based on the realization that it is important to retain the capacity of an adenovirus to counteract host cell apoptosis at least in part in order to prevent premature cell death before adenovirus replication is completed. Therefore, it is preferred that the recombinant adenoviruses of the invention retain one or more genes encoding proteins capable of regulating host cell apoptosis, such as genes of the E1B, E3 and/or E4 region.

[0025] The invention also provides formulations comprising the recombinant adenoviruses according to the invention that can be used to preserve said recombinant adenoviruses and to administer said recombinant adenoviruses to cells. In one variation, the formulations are used to administer said recombinant adenoviruses to cells *in vitro*, in another variation the formulations are used to administer said recombinant adenoviruses to cells *in vivo*.

[0026] The invention furthermore provides methods to administer the formulations according to the invention to cells, leading to infection of said cells with the recombinant adenoviruses of the invention. In one variation, the methods are used to administer said formulations to cells *in vitro*, in another variation the methods are used to administer said formulations to cells *in vivo*.

[0027] The invention also provides compositions of the recombinant adenoviruses according to the invention and cells in which the recombinant adenoviruses according to the invention induce accelerated cell lysis and/or a faster release of virus progeny, compared to recombinant adenoviruses lacking the functional protein according to the invention. In a preferred variation of the invention, said cells are cancer cells and said cell lysis is oncolysis. In a further preferred variation of the invention, said cells are human cells.

[0028] In another embodiment, the invention provides compositions of the recombinant adenoviruses according to the invention and tumors in which the recombinant adenoviruses according to the invention induce accelerated cell lysis and/or a faster release of virus progeny, compared to recombinant adenoviruses lacking the functional protein according to the invention. In this aspect of the invention, it is preferred that said accelerated cell lysis and/or a faster release of virus progeny results

5 in an accelerated lateral spread by said recombinant adenoviruses from infected cells to neighboring cells in said tumors, compared to recombinant adenoviruses lacking the functional protein according to the invention. In this aspect of the invention, it is furthermore preferred that said accelerated cell lysis, faster release of virus progeny and/or accelerated lateral spread lead to a more effective destruction of said tumors. In a preferred variation of the invention, said tumors are growing in an animal body. In a further variation, said animal body is
10 a human body.

[0029] The invention furthermore provides methods to construct the recombinant adenoviruses according to the invention and to produce the formulations and compositions according to the invention.

[0030] The invention furthermore contemplates the use of the recombinant adenoviruses, methods and formulations according to the invention for the treatment of a disease which involves inappropriate cell survival, where it is preferred that said disease is a disease in a
15 human being. In a particular embodiment of the invention said disease is cancer.

[0031] Hereinafter, in several embodiments of the invention a number of ways to provide said recombinant adenoviruses, formulations, methods, compositions, and uses are given. It is to be clearly understood that the description is not meant to in any way limit the scope of the invention as was explained in general terms above. Skilled artisans will be able to transfer the teachings of the present invention to other recombinant adenoviruses, functional proteins, formulations, methods, compositions, and uses that are not mentioned specifically herein without departing from the present invention.

[0032] It is also to be understood that the invention
45 includes all combined uses of the recombinant adenoviruses, formulations, methods and compositions of the invention together with other methods and means to kill a population of cells, including but not limited to irradiation, introduction of genes encoding toxic proteins, such as for example toxins or prodrug converting enzymes, and administration of chemical compounds, antibodies, receptor antagonists, and the like.

[0033] The definitions of the terms used in the invention specification and claims are deemed either to be
55 sufficiently defined herein or otherwise being clearly understood in the art.

Detailed description of the invention

[0034] The invention and preferred embodiments appear from the appended claims. In one embodiment, the present invention provides a recombinant adenovirus, characterized in that it comprises at least one open reading frame for a protein capable of inducing p53-dependent apoptosis functionally linked to regulatory DNA sequences in such a manner that said protein is expressed in a cell into which said recombinant adenovirus is introduced. In a variation of the present invention, a recombinant adenovirus is provided that comprises more than one open reading frame for a protein capable of inducing p53-dependent apoptosis functionally linked to regulatory DNA sequences in such a manner that said protein is expressed in a cell into which said recombinant adenovirus is introduced, where each open reading frame encodes for a different protein capable of inducing p53-dependent apoptosis.

[0035] The virus can be replicated in the target cells in several ways as discussed above; the skilled person will be aware of suitable replication strategies useful for the practise of the invention.

[0036] The recombinant adenovirus according to the invention may e.g. either be (1) a replication competent adenovirus, or (2) a conditionally replicating adenovirus, or (3) a heterologously trans-complemented adenovirus, or (4) a replication deficient adenovirus that is provided *in trans* with a means to replicate in a cell. Said means is understood to include expression in said cell of functions encoded by the parts of the adenovirus genome that were removed from said recombinant adenovirus from stable inserts in the genome of said cell, from DNA constructs introduced into said cell by means known in the art, or from a second recombinant virus used to co-infect said cell, where it is preferred that said recombinant virus is a recombinant adenovirus. In the latter case, the recombinant adenovirus according to the invention together with said second recombinant adenovirus constitutes a two-component replication competent, heterologously trans-complemented, or conditionally replicating adenovirus. Said means is furthermore understood to include expression in said cell of so-called E1A-like activity. Non-limiting examples of conditionally replicating adenoviruses according to the invention are derived from adenoviruses with controlled expression of an essential early adenovirus gene by a tumor-specific promoter, including but not limited to those described by Rodriguez et al. (Cancer Res. 57(1997):2559-2563) and by Hallenbeck et al. (Hum. Gene Ther. 10(1999): 1721-1733) or adenoviruses with mutations in viral genes to abrogate the interaction of the encoded proteins with cellular proteins, necessary to complete the viral life cycle in normal cells, but not in tumor cells, including but not limited to those described by Bischoff et al. (Science 274(1996):373-376) and Fueyo et al. (Oncogene 19(2000):2-12). A non-limiting example of a heterologously trans-complemented adenovirus according

to the invention is derived from a recombinant adenovirus with a functionally deleted E1 region that expresses the HPV E6 and E7 proteins (Steinwaerder et al., Mol. Ther. 4(2001):211-216). For the purpose of the invention, the term "p53-dependent apoptosis" means cell death involving a pathway in which the p53 protein plays a role. Current knowledge on the regulation of programmed cell death and the position of p53 in the apoptotic pathways is reviewed in: Zoernig et al., Biochim. Biophys. Acta 1551(2001):F1-F37; Moll and Zaika, FEBS Letters 493(2001):65-69, included by reference herein. It is to be understood that said protein capable of inducing p53-dependent apoptosis includes the p53 protein itself, as well as its homologues, including but not limited to p63 and p73, and any other currently known or yet to be identified protein member of the p53-dependent apoptosis pathway, including but not limited to BAX, BAK, BOK/Mtd, BCL-X_S, Noxa/APR, PIDD, p53AIP1, PUMA, KILLER/DR5, Apaf-1 and the PIG products (Miyashita and Reed, Cell 80(1995): 293-299; Kiefer et al., Nature 374(1995):736-739; Minn et al., J. Biol. Chem. 271(1996):6306-6312; Polyak et al., Nature 389(1997):300-305; Wu et al., Nature Genet. 17(1997):141-143; Fearnhead et al., Proc. Natl. Acad. Sci. USA 95(1998):13664-13669; Juergensmeier et al., Proc. Natl. Acad. Sci. USA 95(1998):4997-5002; Soengas et al., Science 284(1999):156-159; Oda et al., Science 288(2000):1053-1058; Oda et al., Cell 102(2000): 849-862; Pearson et al., Clin. Cancer Res. 6(2000): 887-890; Lin and Benchimol, Nature Genet. 26(2000): 122-127; Yu et al., Mol. Cell 7(2001):673-682). It is to be understood that it is not necessary that the expression of said protein capable of inducing p53-dependent apoptosis is regulated directly or indirectly by p53 trans-activation. A protein that is capable of potentiating the p53-dependent apoptosis pathway by interacting with one or more members of said pathway is itself also regarded as a member of said pathway. Non-limiting examples of this type of protein capable of inducing p53-dependent apoptosis are BID and its truncated p15 form (tBID) that activate pro-apoptotic bcl-2 family members such as BAX and BAK (Desagher et al., J. Cell Biol. 144(1999):891-901; Wei et al., Genes Dev. 14(2000): 2060-2071; Wei et al., Science 292(2001):727-730), and molecules such as BAD, HRK, Bik/Nbk, and Blk that induce apoptosis by antagonizing survival-promoting bcl-2 family members (Boyd et al., Oncogene 11(1995): 1921-1928; Han et al., Mol. Cell. Biol. 16(1996): 5857-5864; Kelekar et al., Mol. Cell. Biol. 17(1997): 7040-7046; Inohara et al., EMBO J. 16(1997): 1686-1694; Hegde et al., J. Biol. Chem. 273(1998): 7783-7786). Said protein also includes molecules capable of increasing the p53 amount in p53 wild-type cells or capable of trans-activating down-stream effector proteins of p53. Non-limiting examples of proteins capable of increasing the p53 amount in p53 wild-type cells are the protein encoded by the melanoma differentiation associated gene-7 (mda-7) (Jiang et al., Oncogene 11

(1995):2477-2486; Saeki et al., Gene Ther. 7(2000): 2051-2057) and p14ARF in p14ARF-deficient cells. Said protein capable of inducing p53-dependent apoptosis furthermore includes functional variants, analogues, derivates thereof such as mutant proteins and peptides that retain capacity to induce p53-dependent apoptosis. The terms "variant", "analogue" or "derivative" in relation to the above-described proteins or peptides include any substitution, variation, modification, replacement, deletion or addition of one (or more) amino acids from or to the sequence providing the said variant, analogue or derivate, provided that the variant, analogue or derivate retains similar function as compared with the original peptide or protein, i.e. the capacity to induce p53 dependent apoptosis. Such variants, analogues and derivatives are encompassed by the present invention.

[0037] A non-limiting example of a mutant protein that retains capacity to induce p53-dependent apoptosis is the p53 Q22/S23-mutant, which has abrogated growth arrest function but only attenuated apoptosis induction capacity (Venot et al., Oncogene 18(1999):2405-2410). Other non-limiting examples of mutant proteins that retain capacity to induce p53-dependent apoptosis are the p53(d13-19) and CTS1 mutants (Kubbutat et al., Nature 387(1997):299-303; Bougeret et al., Cancer Gene Ther. 7(2000):789-798). The latter mutants lack the binding domain for the MDM2 protein and exhibit enhanced apoptotic activity in cancer cells expressing wild-type p53 (Bougeret et al., Cancer Gene Ther. 7(2000):789-798; Atencio et al., Mol. Ther. 4(2001):5-12). Yet another non-limiting example of a mutant protein that retains capacity to induce p53-dependent apoptosis is a fusion protein of p53 with a mitochondrial import leader peptide (Marchenko et al., J. Biol. Chem. 275(2000): 16202-16212). A non-limiting example of a peptide that retains capacity to induce p53-dependent apoptosis is an amino acid fragment including the Bcl-2 Homology region-3 (BH3) of BAK (Chittenden et al., Nature 374 (1995):733-736; Cosulich et al., Curr. Biol. 7(1997): 913-920; Holinger et al., J. Biol. Chem. 274(1999): 13298-13304). It is preferred that said protein capable of inducing p53-dependent apoptosis is a mammalian protein, for example a human protein, or a functional variant, analogue or derivative thereof. It is furthermore preferred that said protein can interact with and be antagonized by at least one of the adenovirus E1B and/or E4 proteins.

[0038] It is to be understood that "inducing p53-dependent apoptosis" includes active stimulation of the p53-dependent apoptosis pathway as well as restoration of the capacity of a cell to respond to a loss of cell-cycle checkpoint control by undergoing p53-dependent apoptosis.

[0039] In one embodiment, the recombinant adenovirus according to the invention is characterized in that said protein capable of inducing p53-dependent apoptosis is the human p53 protein.

[0040] In a preferred embodiment, the recombinant adenovirus according to the invention is further characterized in that it comprises at least one of the genes of the E1B region. In one variation of this embodiment, said recombinant adenovirus comprises the entire E1B region. In a second variation of this embodiment, said recombinant adenovirus comprises functional expression units for the E1B-19kDa and E1B-55kDa proteins. In a third variation of this embodiment, said recombinant adenovirus comprises a functional expression unit for the E1B-19kDa protein.

[0041] In another preferred embodiment, the recombinant adenovirus according to the invention is further characterized in that it comprises at least one of the genes of the E3 region. In one variation of this embodiment, said recombinant adenovirus comprises the entire E3 region. In a second variation of this embodiment, said recombinant adenovirus comprises functional expression units for one or more of the proteins encoded by the E3 region, such as for example E3-ADP.

[0042] In yet another preferred embodiment, the recombinant adenovirus according to the invention is further characterized in that it comprises the E4orf6 gene.

[0043] Control sequences operably linked to sequences, i.e. the open reading frame, encoding the protein or peptide of interest, include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term promoter is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers. The promoter is typically selected from promoters which are functional in mammalian cells, although promoters functional in other eukaryotic cells may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur. With respect to eukaryotic promoters, they may be promoters that function in a ubiquitous manner (such as promoters of *a*-actin, *b*-actin, tubulin) or, alternatively, a tissue-specific manner (such as promoters of the genes for pyruvate kinase).

[0044] In one variation of the invention, said open reading frame for a protein capable of inducing p53-dependent apoptosis is functionally linked to regulatory DNA sequences in such a manner that said protein is constitutively expressed in a cell into which said recombinant adenovirus is introduced. In this case, the expression of said protein is driven by a constitutive or stable promoter. The present invention does not dictate the choice of said stable promoter. The type of promoter is chosen to accomplish a useful expression profile for said protein in the context of said recombinant adenovirus. Non-limiting examples of useful promoters for this variation of the invention include the Cytomegalovirus (CMV) immediate early promoter, The Simian Virus 40

(SV40) immediate early promoter, and promoters from eukaryotic household genes.

[0045] In another variation of the invention, said open reading frame for a protein capable of inducing p53-dependent apoptosis is functionally linked to one or more control sequences, i.e. regulatory DNA sequences, in such a manner that said protein is only expressed or is expressed at a higher level in a cell into which said recombinant adenovirus is introduced under certain conditions that can be modulated by an external signal, where the term "external" means having its origin outside of the DNA fragment encompassing said open reading frame and said regulatory DNA sequences. In this aspect of the invention, the expression of said protein is driven by a so-called regulatable or inducible promoter. Examples of said external signal include, but are not limited to, the addition or deprivation of a chemical compound, a shift in temperature, a decreased oxygen concentration, irradiation, and the like. Non-limiting examples of this kind of promoter include the heat shock protein 70 promoter, the promoter of an acute phase protein gene, such as the serum amyloid A3 gene or the complement factor 3 gene, the early growth response protein 1 promoter, the multidrug resistance gene 1 promoter, and promoters comprising one or more hypoxia-responsive elements, and fragments thereof (Kohno et al., *Biochem. Biophys. Res. Comm.* 165(1989): 1415-1421; Varley et al., *Proc. Natl. Acad. Sci. USA* 92 (1995):5346-5350; Hallahan et al., *Nature Med.* 1 (1995):786-791; Dachs et al., *Nature Med.* 3(1997): 515-520; Blackburn et al., *Cancer Res.* 58(1998): 1358-1362; Binley et al., *Gene Ther.* 6(1999): 1721-1727; Marples et al., *Gene Ther.* 7(2000): 511-517). A special kind of regulatable promoter is a tissue- or cell type-specific promoter, where said external signal is provided by a protein that is only present in a particular type of cell or tissue. Non-limiting examples of tissue- or cell-type specific promoters are the prostate specific antigen promoter, the alpha-fetoprotein promoter, the albumin promoter, the carcinoembryonic antigen promoter, the cytokeratin 18 gene promoter, the kallikrein 2 promoter, the tyrosinase promoter, the osteocalcin promoter, the PAX-5 promoter and the alpha-lactalbumin promoter (Kaneko et al., *Cancer Res.* 55 (1995):5283-5287; Richards et al., *Hum. Gene Ther.* 6 (1995):881-893; Kozmik et al., *Proc. Natl. Acad. Sci. USA* 92(1995):5709-5713; Siders et al., *Cancer Res.* 56 (1996):5638-5646; Chow et al., *Proc. Natl. Acad. Sci. USA* 94(1997):14695-14700; Shirakawa et al., *Cancer Gene Ther.* 5(1998):274-280; Gotoh et al., *J. Urol.* 160 (1998):220-229; Anderson et al., *Gene Ther.* 6(1999): 854-864; Yu et al., *Cancer Res.* 59(1999):4200-4203). Another special kind of regulatable promoter is a promoter that is responsive to an external signal that is provided by a protein that is not present in a particular type of cell or tissue. In particular, external signals that are absent in liver tissue are of interest in the context of *in vivo* administration of recombinant adenoviruses. Non-

limiting examples of promoters that are responsive to external signals that are absent in liver tissue are the cyclooxygenase-2 promoter and the midkine promoter (Adachi et al., *Cancer Res.* 60(2000):4305-4310;

5 Yamamoto et al., *Mol. Ther.* 3(2001):385-394). Another special kind of regulatable promoter is a promoter that is responsive to an external signal that is provided by a protein that is only present during a certain stage of the cell cycle. A non-limiting example of this kind of promoter 10 is the promoter of a gene that is responsive to E2F, such as for example the adenovirus E2 gene. Another, not mutually excluding, special kind of regulatable promoter is a so-called transactivation response element (TRE). Said TRE is a first component of a transactivation 15 system that comprises as a second component a transactivator protein, that is capable of binding with specificity to said TRE, thereby regulating the transcription of a gene linked to said TRE.

[0046] In yet another variation of the invention, said 20 open reading frame for a protein capable of inducing p53-dependent apoptosis is functionally linked to regulatory DNA sequences in such a manner that said protein is only expressed in a cell into which said recombinant adenovirus is introduced during the late phase of adenovirus replication. Expression of said protein confined to the late phase of adenovirus replication is of particular interest in the context of a CRAd. Since said replication will only occur in cells in which certain conditions exist that are exploited by said CRAd to allow said replication, expression of said protein will also be confined to said cells in which said certain conditions exist. This variation of the invention will thus add to the specificity of said CRAd. In this aspect of the invention, it is preferred that expression of said protein is driven by the 30 adenovirus major late promoter (MLP). In recombinant adenoviruses according to the invention where the MLP drives expression of said open reading frame it is preferred that the expression cassette for said open reading frame comprises the *in cis* acting sequences required 35 to confer full transcriptional activity of the MLP during the late phase of adenovirus replication as defined by Mondesart et al. (*Nucleic Acids Res.* 19(1991): 3221-3228), included by reference herein. A useful expression cassette for this aspect of the invention was 40 disclosed in US5518913, included by reference herein.

[0047] The invention does not dictate the site of insertion of said open reading frame for a protein capable of inducing p53-dependent apoptosis functionally linked to regulatory DNA sequences in the genome of said recombinant adenovirus; said insertion may be at any location in said genome that does not inhibit replication of said recombinant adenovirus in said cell into which said recombinant adenovirus is introduced and where endogenous expression cassettes in said genome do not interfere with proper expression of said open reading frame. In non-limiting examples of the invention, said insertion is a replacement of the adenovirus E3-region or insertion between the E4 promoter and the right-hand 55

ITR. DNA constructs to generate recombinant adenoviruses with insertions in the E3-region are known in the art, including but not limited to pBHG10 and pBHG11 (Bett et al., Proc. Natl. Acad. Sci. USA 91(1994): 8802-8806), and insertions at other sites within the adenovirus genome can be made using standard molecular biology methods known in the art. In another variation of the invention, said open reading frame is inserted in place of an adenovirus gene, where it is preferred that said adenovirus gene is expressed during the late phase of adenovirus replication, and where it is further preferred that said adenovirus gene is functionally linked to the endogenous MLP.

[0048] In one embodiment of the invention, the recombinant adenovirus of the invention is characterized in that: (1) said open reading frame for a protein capable of inducing p53-dependent apoptosis is functionally linked to regulatory DNA sequences in such a manner that said protein is constitutively expressed in a cell into which said recombinant adenovirus is introduced, (2) said recombinant adenovirus comprises at least one of the proteins of the E1B region and of the E4 region, and (3) said protein capable of inducing p53-dependent apoptosis or a direct or indirect down-stream effector protein of said protein capable of inducing p53-dependent apoptosis in the p53-dependent apoptosis pathway can interact with and be antagonized by at least one of the proteins of the E1B region and/or E4 region included in said recombinant adenovirus.

[0049] The recombinant adenoviruses of the invention are produced using molecular biology, virology and cell biology methods known in the art. A way to produce the recombinant adenoviruses according to the invention is described in detail in the examples section. It is to be understood, however, that this description is not meant in any way to limit the scope of the invention. Those skilled in the art will be able to derive the recombinant adenoviruses of the invention using other methods or by using variations of the methods described herein.

[0050] The invention also provides formulations comprising the recombinant adenoviruses according to the invention that can be used to preserve said recombinant adenoviruses and to administer said recombinant adenoviruses to cells. Said formulations preferably consist of said recombinant adenovirus and a diluent. Said diluent allows storage of said recombinant adenovirus for extended time and/or administration of said recombinant adenovirus to cells in culture and/or cells in an animal body, where it is preferred that said animal body is a human body. It is preferred that said diluent allows storage under lyophilized conditions. It is also preferred that said diluent allows both storage and administration of said recombinant adenovirus to cells in culture and/or cells in an animal body. It is to be understood that "to allow storage" means that during storage of said formulation the capability of said recombinant adenovirus to infect a cell is retained with a half-life higher than one

week, where it is preferred that said half-life is more than one month, and where it is most preferred that said half-life is more than 6 months. Said storage may be at any temperature below 40°C, but it is preferred that said temperature is between 1°C and 10°C, or that said temperature is below minus 60°C. It is to be understood that said administration to cells in culture and/or cells in an animal body means that said formulation and said cells are brought together resulting in introduction of said recombinant adenovirus into said cells. It is preferred that said diluent is not toxic to said cells and to said animal body. The invention does not dictate the exact composition of said diluent, but several useful diluents for the purpose of the invention are known in the art. Non-limiting examples of diluents useful in the invention include buffer solutions based on phosphate, such as PBS, or Tris or HEPES at a concentration between 10 and 25 mM and with a pH between 7.0 and 8.0, containing up to 150 mM NaCl or a combination of NaCl and KCl, and between 1 and 10 mM MgCl₂ or a combination of MgCl₂ and CaCl₂, with up to 3% sucrose or up to 40% glycerol. Other non-limiting examples of diluents according to the invention include the standard culture media for said cells known in the art, such as for example DMEM, IM-DM or RPMI-1640, optionally supplemented with animal serum or serum components or recombinant serum proteins, chemically defined culture medium, including protein-free medium, and pharmaceutical diluents known in the art for administration of drugs into an animal body, such as for example Haemaccel (Behring Pharma). Optionally, said diluent may be further supplemented with additional constituents to increase physical stability of said recombinant adenovirus during storage or to increase said introduction into said cells. Said constituents may be different for each specific use of said formulation. Non-limiting examples of said additional constituents are polycations (Arcasoy et al., Gene Ther. 4 (1997):32-38; Kaplan et al., Hum. Gene Ther. 9(1998): 1469-1479; Lanuti et al., Gene Ther. 6(1999): 1600-1610), polycationic polymers and cationic lipids (Fasbender et al., J. Biol. Chem. 272(1997):6479-6489; Qiu et al., Hum. Gene Ther. 9(1998):507-520), polyamide compounds (Connor et al., Gene Ther. 8(2001): 41-48), and the ingredients disclosed by Croyle et al (Mol. Ther. 4(2001):22-28). Optionally, said diluent may be further supplemented with additional constituents to improve the administration of said recombinant adenoviruses to said cells in an animal body. Non-limiting examples of such constituents are compounds that increase the permeability of cell layers in the wall of a blood vessel, such as for example bradykinin, serotonin and RMP-7 (Donahue et al., Gene Ther. 5(1998): 630-634; Rainov et al., Hum. Gene Ther. 10(1999): 311-318), or proteases that are active against extracellular matrix proteins (Kuriyama et al., Cancer Res. 61 (2001):1805-1809), such as for example collagenases, gelatinases, matrilysin, stromelysines, dispase, trypsin, neuraminidase, serine proteases, and the like. If said

cells in an animal body are cancer cells in a solid tumor, than it is preferred that said proteases are active against extracellular matrix proteins that are present in said tumor more abundantly than in other parts in said animal body, or that said protease is activated by proteins that are present more abundantly in said tumor than in other parts of said animal body. Non-limiting examples of such proteins that are present more abundantly in said tumor than in other parts of said animal body and that are capable of activating said proteases are membrane type metalloproteases (MT-MMPs) (Seiki, A.P.M.I.S. 107 (1999):137-143). Those skilled in the art will be able to define by proper investigation useful diluents to prepare a formulation according to the invention that results in the introduction of said recombinant adenovirus into cells for each particular use of the invention and each particular method of administration according to the invention.

[0051] The invention furthermore provides methods to administer the formulations according to the invention to cells, leading to introduction of the recombinant adenoviruses of the invention into said cells. In one variation, the methods are used to administer said formulations to cells *in vitro*, in another variation the methods are used to administer said formulations to cells *in vivo*. The methods according to the invention do not differ in any way from those known in the art to administer other recombinant adenoviruses to cells. In general, the recombinant adenoviruses of the invention are diluted to reach a useful concentration in a diluent according to the invention. In general, said diluent is isotonic to the conditions in an animal body, but in some cases it may be desired to use a diluent at a non-isotonic concentration. Said MgCl₂, CaCl₂, sucrose and glycerol are not required, and in the case of *in vivo* administration it is preferred that the concentration of glycerol is as low as possible. Said useful concentration of said recombinant adenovirus will be different for each different use of the invention. Skilled artisans will be able to determine said useful concentration by experimentation. Said formulation is brought into contact with said cells under either static conditions, such as in the case of administration to cells in culture or in the case of injection into an animal tissue, or under dynamic conditions, such as in the case of injection into the blood circulation of an animal body. Said formulation and said cells are brought into contact at a temperature between 0°C and 50°C, where it is preferred that said temperature is between 30°C and 40°C. In case said formulation is administered into an animal body, it is preferred that said formulation and said cells are brought into contact at the existing temperature in said animal body. In one variation of the invention, said administration is done at an ambient atmospheric pressure. In another variation of the invention, said administration is done at a pressure above atmospheric pressure. Said contact is maintained for a time period sufficiently long to allow introduction of said recombinant adenoviruses into said cells.

[0052] The invention also provides compositions of a recombinant adenovirus that comprises at least one open reading frame for a protein capable of inducing p53-dependent apoptosis according to the invention and cells in which said recombinant adenovirus replicates. Said recombinant adenoviruses have a host range that allows replication in said cells. Said composition results in at least one of the two following situations, i.e., (1) said cells are lysed more rapidly than when said cells are combined with a recombinant adenovirus other than the recombinant adenovirus according to the invention, and/or (2) virus progeny of said recombinant adenovirus according to the invention is released faster from said cell than virus progeny of a recombinant adenovirus other than the recombinant adenovirus according to the invention is released from said cell, where it is preferred that said composition results in both situations. In a preferred variation of the invention, said cells are cells that have lost capacity to respond to a loss of cell-cycle checkpoint control by undergoing p53-dependent apoptosis. In particular examples of this variation of the invention, said cells are rheumatoid arthritis cells or cancer cells. For the purpose of the invention, the terms "cancer cells" and "tumor cells" are defined as cells, having lost proper cell growth control, leading to uncontrolled growth and/or replication of the said cells in e.g. a mammalian body, or to accelerated growth/replication or immortality *in vitro*. Thus, the term includes malignant, premalignant and benign cancer cells. In a not mutually excluding preferred variation of the invention, said cells are human cells. In another not mutually excluding variation of the invention, said cells are cells in an animal body, where it is preferred that said animal body is a human body. The compositions of the invention are obtained by administering a formulation containing a recombinant adenovirus according to the invention to said cells by means of a method according to the invention.

[0053] In one embodiment of the invention, said cells that are part of a composition according to the invention, are cells in a solid tumor. In one variation of this embodiment, said tumor is maintained in culture *in vitro*. In this variation of the invention, said tumor may be artificially derived from cancer cells, such as for example a cell line-derived spheroid, or said tumor may be derived from an explant of a tumor in an animal body. In another variation of this embodiment, said tumor is present in an animal body. In this variation of the invention, said tumor may be surgically implanted into said animal body or said tumor may have arisen from said animal body. In the latter case, it is preferred that said animal body is a human body. In this embodiment of the invention, it is preferred that the more rapid cell lysis and/or faster release of virus progeny results in an accelerated lateral spread by said recombinant adenoviruses from infected cells to neighboring cells in said tumor, compared to recombinant adenoviruses lacking the protein capable of inducing p53-dependent apoptosis according to the in-

vention. In this aspect of the invention, it is furthermore preferred that said more rapid cell lysis, faster release of virus progeny and/or accelerated lateral spread lead to a more effective destruction of said tumor.

[0054] The invention furthermore contemplates the use of the recombinant adenoviruses, methods and formulations according to the invention for the treatment of a disease which involves inappropriate cell survival, where it is preferred that said disease is a disease in a human being. A treatment according to the invention will comprise administration of a recombinant adenovirus according to the invention, in a formulation according to the invention, to diseased cells in an animal body using a method according to the invention. In a particular embodiment of the invention said disease is cancer and said diseased cells are cancer cells, where it is preferred that said cancer cells are part of a solid tumor or a tumor metastasis. Depending on the type of disease and the nature of the diseased cells, a useful recombinant adenovirus, a useful formulation and a useful route of administration will be chosen. With respect to said recombinant adenovirus, a useful protein capable of inducing p53-dependent apoptosis may be chosen on the basis of prior investigation, but preferably also on the basis of knowledge of the genetic background of said disease in general, or more preferably of the genetic background of said diseased cells in particular. A useful formulation and route of administration will be chosen on the basis of knowledge on the localization of said diseased cells in said animal body, the characteristics of said diseased cells and the characteristics of other cells present in the part of said animal body to which said formulation is administered. Non-limiting examples of said route of administration include direct injection into a tissue containing diseased cells, oral administration, injection into the blood circulation, inhalation, injection into a body cavity, such as the pleural or peritoneal cavity, a joint, or a brain ventricle, injection into the lumen of a part of the gastrointestinal or urogenital tract, and application to the surface of a certain body area, such as the skin or the otolaryngeal mucosa, for example by means of a mouth wash. If said route of administration is via injection into the blood circulation, it is preferred that said injection is done into an artery that leads to a part of said animal body that contains said diseased cells.

[0055] The invention furthermore contemplates that a treatment of a disease according to the invention is combined with other methods and means to kill a population of diseased cells known in the art, including but not limited to irradiation, introduction of genes encoding toxic proteins, such as for example diphtheria toxin, or prodrug converting enzymes like thymidine kinase, cytosine deaminase or carboxylesterase, or cytokines like interleukin-2 or GM-CSF, or anti-angiogenic products like endostatin or angiostatin and administration of chemical compounds, antibodies, receptor antagonists, and the like. It is anticipated that such a combined treatment may result in a more effective killing of said population

of diseased cells than either treatment alone. In addition, one treatment may potentiate the effect of the other treatment. For example, irradiation and certain chemical compounds are known to induce the p53-dependent apoptosis pathway. Thus, such treatments may potentiate the efficient cell lysis and virus progeny release of a recombinant adenovirus according to the invention.

[0056] Hereinafter, in several examples a number of ways to provide said recombinant adenoviruses, formulations, methods, compositions, and uses according to the invention are described in more detail. It is to be clearly understood that the description is not meant to in any way limit the scope of the invention as was explained in general terms above. Skilled artisans will be able to transfer the teachings of the present invention to other recombinant adenoviruses, functional proteins, formulations, methods, compositions, and uses without departing from the present invention.

20 Examples

Example 1. Production of replication-competent and conditionally replicating adenoviruses expressing the human tumor suppressor protein p53 and control adenoviruses without p53.

[0057] To construct adenoviruses with an expression cassette for human p53 in the E3 region, the SVE-p53 expression cassette (SV40 early promoter-driven human p53 cDNA including intron-4) was released from pAdHumPwt.SVE (Ameyar et al., *Oncogene* 18(1999): 5464-5472) by digestion with KpnI and XbaI (partial). The 2.6 kb fragment was inserted into KpnI/XbaI-digested pABS.4 (Microbix Biosystems, Toronto, Canada). The resulting construct was designated pABS.4-p53. pABS.4-p53 was digested with PstI and the 4 kb fragment carrying the SVE-p53 cassette and kanamycin resistance gene was inserted into PstI-digested pBHG1 (Microbix Biosystems). A clone with an insert in the orientation that places the SVE-p53 cassette on the adenovirus 1-strand was isolated and designated pBHG11-p53kan-L. The kanamycin resistance gene was removed by digestion with SmaI followed by self-ligation, yielding pBHG11-p53-L.

[0058] Replication competent adenoviruses were made by homologous recombination in 293 cells (Graham et al., *J. Gen. Virol.* 36(1977):59-72) between pXC1 (Microbix Biosystems) or pXC1-derivatives with E1 mutations rendering the vectors conditionally replicating together with pBHG11 or pBHG11-p53-L. The pXC1-derivatives were pXC1-Δ24, carrying a 24 bp deletion in the pRb-binding CR2 domain in E1A (Fueyo et al., *Oncogene* 19(2000):2-12) and pXC1-Δ55K carrying a deletion from the Sau3AI site at adenovirus serotype 5 (Ad5) nt 2426 to the BglII site at Ad5 nt 3328 encompassing a large part of the E1B-55kDa protein open reading frame. This way, the following viruses were made: AdE1 with wild-type E1 region, AdΔ24 with the

E1A CR2-mutation, AdΔ55K with the E1B-55kDa protein-deletion, and the three p53-expressing derivatives AdE1-p53, AdΔ24-p53, and AdΔ55K-p53.

[0059] Viruses were plaque purified, propagated on A549 lung carcinoma cells (obtained from the ATCC, Manassis, VA), and CsCl gradient purified according to standard techniques. The E1 mutations and SVE-p53 insertion were confirmed by PCR on the final products. Expression of p53 protein was confirmed by infection of SaOs-2 p53-null cells (obtained from Dr. F. van Valen, Westfälische Wilhelms-Universität, Munster, Germany) followed by Western analysis with anti-p53 MoAb DO-7 (Dako, Glosdrup, Denmark). Functional expression of p53 protein resulting in transactivation of p53-dependent promoters was confirmed by infection of SaOs-2 cells that were transfected with the p53-dependent reporter plasmid PG13-Luc (El-Deiry et al, Cell 75(1993) 817-825) and measurement of luciferase expression. Particle titers of all adenoviruses were determined by OD260 measurement and functional plaque forming unit (pfu) titers were determined by limiting dilution plaque titration on 293 cells according to standard techniques.

Example 2. Expression of functional p53 protein in adenovirus-infected cells enhances cell lysis.

[0060] To demonstrate that expression of a functional component of the p53-dependent apoptosis pathway during adenovirus replication augments lysis of the host cell, a dual-virus system was used in which cells were infected with equal amounts of the replication-competent adenovirus AdE1+Luc (a kind gift of Dr. R. Vogels, Crucell Holland BV, Leiden, The Netherlands) that was derived from wild-type Ad5 through replacement of the gp19k open reading frame in the E3 region by the firefly luciferase gene, and the replication-defective vector Adp53 (Ameyar et al., Oncogene 18(1999):5464-5472), expressing human wild-type p53 protein. This dual-virus system creates a situation where p53 is expressed in the context of a replicating adenovirus. As a negative control, Adp53 was replaced by the irrelevant control vector AdGFP (expressing CMV promoter-driven Enhanced Green Fluorescence Protein; van Beusechem et al., Gene Ther. 7(2000):1940-1946). In further control cultures, cells were infected with AdGFP or Adp53 only, to investigate the effect of apoptosis induction or growth arrest by p53 *per se*. A panel of human cancer cell lines with different p53 status was subjected to (dual) virus infection and cell viability was monitored over a two-week period. The cell lines included were: SaOs-2 osteosarcoma cells, that carry a homozygous p53 gene deletion (Masuda et al., Proc. Natl. Acad. Sci. USA 84 (1987):7716-7719); HT-29 colon carcinoma and U373MG glioma cell lines, that carry a codon 273 R to H mutation in their p53 gene (Van Meir et al., Cancer Res. 54(1994):649-652; Rodrigues et al., Proc. Natl. Acad. Sci. USA 87(1990):7555-7559); OVCAR-3 ovary

carcinoma cells, that carry a codon 248 R to Q mutation in their p53 gene (Yaginuma and Westphal, Cancer Res. 52(1992):4196-4199); and A549 lung carcinoma cells, that express wild-type p53 protein (Lehman et al., Cancer Res. 51(1991):4090-4096). SaOs-2 cells were obtained from Dr. F. van Valen, Westfälische Wilhelms-Universität, Munster, Germany; all other lines were purchased from the ATCC, Manassis, VA, USA. All cell lines were seeded $5 \cdot 10^4$ cells per well in 24-well plates in

10 F12-DMEM/10% FCS and cultured overnight. The next day, dual-virus mixtures with equal pfu titers were prepared of AdE1+Luc with AdGFP or AdE1+Luc with Adp53 in F12-DMEM with 2% FCS. The mixtures were used to infect SaOs-2 cells at a multiplicity of infection (MOI) of 50 pfu/cell; A549 and U373MG cells at 100 pfu/cell; and OVCAR-3 and HT29 cells at 500 pfu/cell of each virus for 1 hour at 37°C. The cells were then washed once with 1 ml F12-DMEM/10% FCS and subsequently cultured in 0.5 ml F12-DMEM/10% FCS at 15 37°C for up to 14 days. This procedure resulted in an efficient infection, as evidenced by detectable GFP expression in most of the cells infected with AdGFP two days after infection. Thus, many cells contained both viruses allowing the E1 proteins provided by AdE1+Luc 20 to trans-complement Adp53 or AdGFP replication. At several time-points, cell viability was determined by removing the culture medium and replacing it by 200 µl 10% WST-1 reagent (Roche Diagnostics, Mannheim, Germany) in culture medium. Depending on the cell type 25 and density, the formation of the formazan dye was allowed to proceed for 30-60 minutes at 37°C. One hundred µl WST-1 medium was transferred to a 96-well ELISA plate (Greiner, Frickenhausen, Germany) and the OD₄₅₀ was measured. WST-1 conversion was expressed as a percentage of the conversion by uninfected control cells, after subtraction of background values 30 of WST-1 incubated in the absence of cells.

[0061] As can be seen in figure 1, AdE1+Luc/AdGFP-induced cell killing was severely delayed in p53-deficient SaOs-2, HT-29 and OVCAR-3 cell lines compared to p53 wild type A549 lung carcinoma cells. The p53-mutant U373MG cell line showed an intermediate rate of adenovirus-induced cell death. Adp53 infection alone affected p53-deficient SaOs-2 and OVCAR-3 cell 40 lines to various degrees during the first few days after infection, but had no significant effect on the viability of p53 wild-type A549 cells and p53-mutant HT-29 or U373MG cells. Moreover, all Adp53-infected cultures 45 fully recovered during the course of the experiment, showing that functional p53 expression alone does not result in an effective means to kill cancer cells. Importantly, AdE1+Luc/Adp53-infected cells were killed much faster and more effectively than control AdE1+Luc/ AdGFP-infected cells. Expression of functional p53 accelerated the killing of all cell lines except HT-29 by at 50 least 3 days, irrespective of the cell line p53 status. Strikingly, OVCAR-3 cells actually required wild-type p53-expression to be sensitive to adenovirus-induced cell 55

death at all. Hence, on most cancer cell lines tested, the combination of adenovirus replication and wild-type p53 expression caused the fastest and most effective cell death.

Example 3. Expression of functional p53 protein in adenovirus-infected cells accelerates the release of progeny virus.

[0062] To demonstrate that the augmented cell lysis due to expression of a functional component of the p53-dependent apoptosis pathway during adenovirus replication results in an earlier release of virus progeny, the same dual-virus infection experiment was performed as described in example 2 and at various time-points during the 14-day culture period the AdE1+Luc virus titer was determined in the culture medium of dual-virus infected cells. To this end, the culture medium was harvested and cleared by centrifugation. The cell-free supernatant was serially diluted in F12-DMEM/10% FCS and used to infect A549 cells seeded 10^4 cells/well in 96-well plates 24 hours before infection. A control titration of AdE1+Luc virus with known pfu titer was included. After 20-24 hours, the culture medium was replaced by Luciferase Chemiluminescent Assay System Reporter Lysis Buffer (Promega, Madison, WI, USA) and the culture plates were subjected to a single freeze/thaw cycle. Chemiluminescence was measured with a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany) during the 10 seconds immediately after addition of the cell lysate to the Luciferase Assay Reagent. Values in the linear range of the serial dilution were used to calculate the luciferase infectious unit (IU) titer. This assay was linear over 3-4 orders of magnitude, with a threshold of approximately 10^3 pfu.

[0063] Figure 2 shows that the virus release rate of control AdE1+Luc/AdGFP-infected cells correlated with the p53 status of the cell. Three days after infection, p53 wild-type A549 cells had already shed more than 10^7 IU into the medium, while p53-null SaOs-2 cells showed 250-fold lower titers and mutant p53-expressing cells produced intermediate amounts. Introduction of functional p53 by means of AdE1+Luc/Adp53 infection enhanced the virus titers in the medium of all cell lines shortly after infection (with a range of 3-20-fold), irrespective of the cell line p53 status. In the case of SaOs-2 cells, where the p53-dependent lysis enhancement was most prominent, the acceleration of virus release was at the expense of a decrease in the total amount of virus produced over the 14-day period. The fast death of AdE1+Luc/Adp53-infected SaOs-2 cells lowered the total virus production approximately 100-fold in the experiment shown in figure 2 and approximately 20-fold in an independent second experiment. The virus production by the other cell lines was not negatively influenced by wild-type p53 introduction. In contrast, expression of functional p53 enhanced the total virus output in the medium of HT-29 and U373MG cells.

[0064] The faster accumulation of infectious virus particles in the culture medium could be the result of an accelerated virus production and/or an earlier virus release. To assess the cause for the observed titer differences

5 early after infection, the AdE1+Luc virus titer was determined on cell lysates as well as on culture media of infected cells 3 days after infection. To this end, the culture medium was harvested and cleared by centrifugation. The cell-free supernatant was used to measure the
10 titers of released AdE1+Luc virus. Non-adherent cells collected by centrifugation and adherent cells scraped from the culture plate were combined, resuspended in culture medium and subjected to three freeze/thaw cycles. The lysate was used to measure the
15 AdE1+Luc virus titer inside the cells. The same method to determine the AdE1+Luc virus titer was used as described above. This experiment was performed on the same set of cell lines as above, and on two additional p53 wild type cancer cell lines, i.e., H460 large cell lung
20 carcinoma cells (cultured in RPMI-1640/10% FCS) and MCF-7 breast carcinoma cells.

[0065] As can be seen in figure 3A, the amount of functional virus produced in infected cells within 3 days was not significantly affected by p53 expression (average 2.3-fold increased on different cell lines; range from a 4-fold decrease on SaOs-2 to a 7-fold increase on HT-29), while the titer in the medium was increased by p53 expression for all cell lines (average 45-fold increased; range from 1.6-fold on OVCAR-3 to 143-fold on A549).
25 In AdE1+Luc/AdGFP-infected cultures, only 0.05-2.1% of the total virus progeny was released within three days (see figure 3B). Expression of p53 strongly augmented the virus release (average 20.4-fold; range 2.8-fold to 68-fold), resulting in 1.1-14.5% of the total virus already
30 released within three days (see figure 3B). Hence, p53 expression during adenovirus replication reproducibly augmented the early release of virus progeny from the cell.

[0066] For the treatment of cancer, conditionally replicating adenoviruses (CRAds) are of particular interest. CRAds differ from replication-competent adenoviruses by specific mutations in their genome. Two important types of CRAds are one that lacks expression of the

50 E1B-55kDa protein and one that produces mutant E1A proteins incapable of binding to pRb. To investigate if the invention is relevant in the context of these CRAds, SaOs-2 cancer cells were infected with dual-virus mixtures consisting of the following components: (1) AdE1 (with wild type E1 region) or AdΔ24 (with a deletion in the pRb-binding CR2 domain in E1A) or AdΔ55K (with a large deletion in the E1B-55kDa protein open reading frame) at an MOI of 30, 10, or 1 pfu/cell, together with

(2) AdGFP or Adp53 at an MOI of 30 pfu/cell. The MOIs were chosen such that most cells would be infected with component (2), and also with component (1) at the highest MOI used, but at lower MOI not all cells would initially be infected with component (1). In the latter case, more than one cycle of adenovirus replication is required to eradicate the entire cell population.

[0067] The same method to administer the viruses to the cells was used as described in example 2. Five or six days after infection, the culture medium was removed and the adherent cells were fixed for 10 minutes at room temperature with 4% formaldehyde in PBS and subsequently stained using 1% crystal violet dye in 70% ethanol for 20 minutes at room temperature. After several washes with water the culture plates were air-dried. The result of this experiment is shown in figure 4. The crystal violet dye stains all remaining cells on the surface of the culture dish. Thus, less staining indicates that more cells have been killed by the recombinant adenoviruses. From this experiment the following conclusions could be drawn: (1) all three recombinant adenoviruses AdE1, AdΔ24 and AdΔ55K were capable of killing SaOs-2 cells, (2) in the absence of p53 protein expression, none of the three viruses was capable of completely eradicating the SaOs-2 cell monolayer during the span of the experiment, and (3) expression of functional p53 protein strongly enhanced the killing of SaOs-2 cells by all three recombinant adenoviruses, showing that (4) expression of p53 increased the number of completed lytic replication cycles by AdE1, AdΔ24 and AdΔ55K within the span of the experiment. These findings were confirmed in several independent experiments using dual-virus mixtures at various MOI on SaOs-2, A549 and U373MG cells. An example of such an experiment is shown in figure 5. Ten to 100-fold enhanced oncolysis was observed when p53 was expressed. The enhancement was similar for the three tested viruses AdE1, AdΔ24 and AdΔ55K. In conclusion, expression of a functional component of the p53-dependent apoptosis pathway enhances lysis of cancer cells by replication-competent recombinant adenoviruses as well as by conditionally replicating recombinant adenoviruses. Thus, the specific mutations that are introduced into the adenovirus genome to construct CRAds do not interfere with the potentiation of oncolysis by a functional component of the p53-dependent apoptosis pathway. The accelerated cancer cell lysis and concomitant accelerated virus release, allowing faster virus spread through the cancer cell population, due to p53 expression resulted in a faster destruction of the entire cancer cell population. This further underlines the relevance of the invention for applications in the area of cancer treatment.

Example 5. Conditionally replicating adenoviruses expressing functional p53 show enhanced oncolytic potency on cancer cells.

[0068] To investigate if the enhanced cell lysis and ac-

celerated virus progeny release due to expression of functional p53 could also be accomplished if the p53 protein is expressed from a stable insert in the genome of a CRAd, the oncolytic potency of the p53-expressing

5 CRAd AdΔ24-p53 was compared to that of the parental CRAd AdΔ24. A549, U373MG and SaOs-2 cells were seeded 5.10⁴ cells per well in 24-well plates in F12-DMEM/10% FCS and cultured overnight. The next day, the cells were infected with AdGFP or Adp53 or 10 AdΔ24 or AdΔ24-p53, diluted in F12-DMEM with 2% FCS to reach an MOI of 100 pfu/cell, 10 pfu/cell, 1 pfu/cell, 0.1 pfu/cell, or 0.01 pfu/cell, for 1 hour at 37°C. The virus-containing medium was then replaced by 1 ml F12-DMEM/10% FCS and cells were cultured at 37°C. 15 After one week, 50% of the culture medium was refreshed. After 12 days, the culture medium was removed and the adherent cells were stained with crystal violet as described in example 4. As can be seen in figure 6, the control virus AdGFP had no effect on the viability of the cells. Adp53 virus only had a very modest inhibitory effect on SaOs-2 cells at the highest MOI. AdΔ24 exhibited a dose-dependent lytic activity on all three cell lines. Most importantly, AdΔ24-p53 killed all 20 three cancer cell lines more effectively than did AdΔ24. 25 An approximately 100, more than 100, or 10-fold lower titer of AdΔ24-p53 than of AdΔ24 was required to accomplish similar killing potency on A549, U373MG and SaOs-2 cancer cells, respectively. Hence, expression of a functional component of the p53-dependent apoptosis 30 pathway from a stable insert in the genome of a conditionally replicating recombinant adenovirus enhanced the oncolytic potency of said recombinant adenovirus 10-100-fold. The oncolytic potency was enhanced irrespective of the cancer cell p53 genetic background.

35 **[0069]** In independent experiments, a larger panel of human cancer cell lines was subjected to the same analysis for oncolytic potency of AdΔ24-p53 versus AdΔ24. Expression of p53 from a stable insert in the AdΔ24 genome resulted in enhanced oncolytic potency on A431 40 epithelial carcinoma cells; U373MG brain cancer cells; MDA-MB-231 breast carcinoma cells; HeLa cervix carcinoma cells; A549 and H460 lung carcinoma cells; OVCAR-3 ovary carcinoma cells; 11B and 22A head and neck squamous carcinoma cells; PC-3 prostate carcinoma cells; HepG2 liver cancer cells; SW1398, Colo205 and HT-29 colon carcinoma cells; and HM02 and MKN28 gastric carcinoma cells. Thus, the invention has 45 broad application for a variety of cancer types.

50 **Description of the figures**

[0070] Figure 1. Killing of human cancer cells by recombinant adenovirus replication is enhanced by p53 expression. Five human cancer cell lines (as indicated 55 in the panels) with different p53 status were infected with AdGFP (open circles), Adp53 (closed circles), AdE1+Luc/AdGFP dual-virus mixture (open squares), or AdE1+Luc/Adp53 dual virus mixture (closed

squares), and cultured up to 14 days. At various time-points, the cell viability was determined by WST-1 conversion assay and compared to the viability of uninfected control cultures. Data shown are the average percentages of viable cells +/- standard deviations of triplicate infections.

[0071] Figure 2. Release of recombinant adenovirus progeny from infected human cancer cells is enhanced by p53 expression. Five human cancer cell lines (as indicated in the panels) with different p53 status were infected with AdE1+Luc/AdGFP dual-virus mixture (open circles), or AdE1+Luc/Adp53 dual virus mixture (closed squares), and cultured up to 14 days. At various time-points, the AdE1+Luc virus titer in the cell-free culture medium was determined on A549 cells. Data shown are the average virus titers +/- standard deviations of triplicate cultures.

[0072] Figure 3. Early release of recombinant adenovirus progeny from infected human cancer cells is augmented by p53 expression. Seven different human cancer cell lines were infected with AdE1+Luc/AdGFP dual-virus mixture, or AdE1+Luc/Adp53 dual virus mixture, and cultured for 3 days. After three days, the cell-free culture medium and the cells were harvested separately and the AdE1+Luc virus titer in these fractions was determined on A549 cells. Panel A shows the average virus titers + standard deviations of five infected cultures, determined on AdE1+Luc/AdGFP infected cells (gray bars), AdE1+Luc/Adp53 infected cells (black bars), the medium of AdE1+Luc/AdGFP infected cells (gray/black hatched bars), or the medium of AdE1+Luc/Adp53 infected cells (white/black hatched bars). Panel B gives the average percentages + standard deviations of virus released within 3 days, relative to the total amount of virus present inside cells and in the culture medium, calculated from the data in panel A, for AdE1+Luc/AdGFP infected cultures (gray bars) and AdE1+Luc/Adp53 infected cultures (black bars).

[0073] Figure 4. Killing of SaOs-2 human osteosarcoma cells by conditionally replicating adenoviruses (CRAds) is enhanced by p53 expression. SaOs-2 cells were infected with AdGFP (upper row in each panel) or Adp53 (lower row in each panel) at MOI 30 pfu/cell, alone (first column in each panel) or together with AdE1 (second column in each panel), AdΔ24 (third column in each panel), or AdΔ55K (fourth column in each panel) at MOI 30 pfu/cell (panel A), MOI 10 pfu/cell (panel B), or MOI 1 pfu/cell (panel C). After five days (panels A and B) or six days (panel C) culture, adherent cells were stained with crystal violet and scanned. Staining is a semi-quantitative measure for the amount of viable cells.

[0074] Figure 5. Killing of A549 (A), SaOs-2 (B) and U373MG (C) cells by conditionally replicating adenoviruses (CRAds) is enhanced by p53 expression. Cells were infected with AdGFP (columns marked +G) or Adp53 (columns marked +P) at MOI 100 pfu/cell, alone (first row in each panel) or together with AdΔ55K (first

two columns in each panel), AdΔ24 (third and fourth column in each panel), or Ade1 (last two columns in each panel) at MOI 0.1, 1, or 10 pfu/cell as indicated. After ten days, adherent cells were stained with crystal violet and scanned. Staining is a semi-quantitative measure for the amount of viable cells.

[0075] Figure 6. A conditionally replicating adenovirus expressing p53 kills human cancer cells more rapidly than the parental control virus not expressing p53. A549 (panel A), SaOs-2 (panel B) and U373MG (panel C) cells were infected with AdGFP (first row in each panel), Adp53 (second row in each panel), AdΔ24 (third row in each panel), or AdΔ24-p53 (fourth row in each panel), at an MOI dilution titration ranging from 100 pfu/cell to 0.01 pfu/cell as indicated above the panels. After 12 days, adherent cells were stained with crystal violet and scanned. Staining is a semi-quantitative measure for the amount of viable cells. Data shown are a representative example of three independent experiments performed.

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Claims

1. Recombinant virus, being capable to replicate in target cells, and having lytic capacity in the said cells, the virus comprising in the genome thereof, the coding sequence of at least one protein functional in restoring the p53 dependent apoptosis pathway in the said target cells, operably linked to one or more expression control sequences, functional in the said target cells.
2. Recombinant virus according to claim 1, wherein the virus is an adenovirus, preferably human adenovirus preferably of serotype 5.
3. Recombinant virus according to claims 1 or 2, wherein the protein functional in restoring the p53 dependent apoptosis pathway is chosen from the group, consisting of p53, p63, p73, BAX, BAK, BOK/Mtd, BCL-X_S, Noxa/APR, PIDD, p53AIP1, PUMA, KILLER/DR5, Apaf-1, PIG, BID, tBID, BAD, HRK, Bik/Nbk, BLK, mda-7, p14ARF or a functional variant, analogue or derivative thereof.
4. Recombinant virus according to claim 3, wherein the protein is p53 or a functional analogue or derivative thereof, preferably being human p53.
5. Recombinant virus according to any of the claims 1-4, wherein the virus genome comprises one or more genes, encoding proteins capable of regulating the apoptosis of the target cells, preferably chosen from the group consisting of genes of the adenovirus E1B, E3 and E4 region or combinations thereof.
6. Recombinant virus according to any of the preced-

ing claims, being capable to replicate in target cells, hampered in the p53 dependent apoptosis pathway.

7. Recombinant virus according to claim 6, wherein the target cell is a human cell, preferably chosen from the group, consisting of cancer cells, arthritic cells, hyperproliferative vascular smooth muscle cells and cells infected with a virus other than the said recombinant virus. 5

8. Recombinant virus according to any of the claims 1-7 for use as a medicament. 10

9. Use of a recombinant virus according to any of the claims 1-7 for the manufacture of a medicament for suppressing uncontrolled cell growth, in particular malignant cell growth. 15

10. Use of a recombinant virus according to claim 9, wherein the target cells are chosen from the group, consisting of cancer cells, arthritic cells, hyperproliferative vascular smooth muscle cells and cells infected with a virus other than the said recombinant virus. 20

11. Method for lysing target cells hampered in the p53 dependent apoptosis pathway, comprising the steps of: 25

- infecting the said target cells with a virus, having lytic capacity in the said target cells, preferably a virus according to any of the claims 1-7, 30
- replicating the said virus within the said target cells, further comprising the step of providing, in the virus genome the coding sequence of at least one protein, functional in restoring the p53 dependent apoptosis pathway, the said coding sequence being capable to be expressed in the target cells upon infection thereof by the said virus. 35

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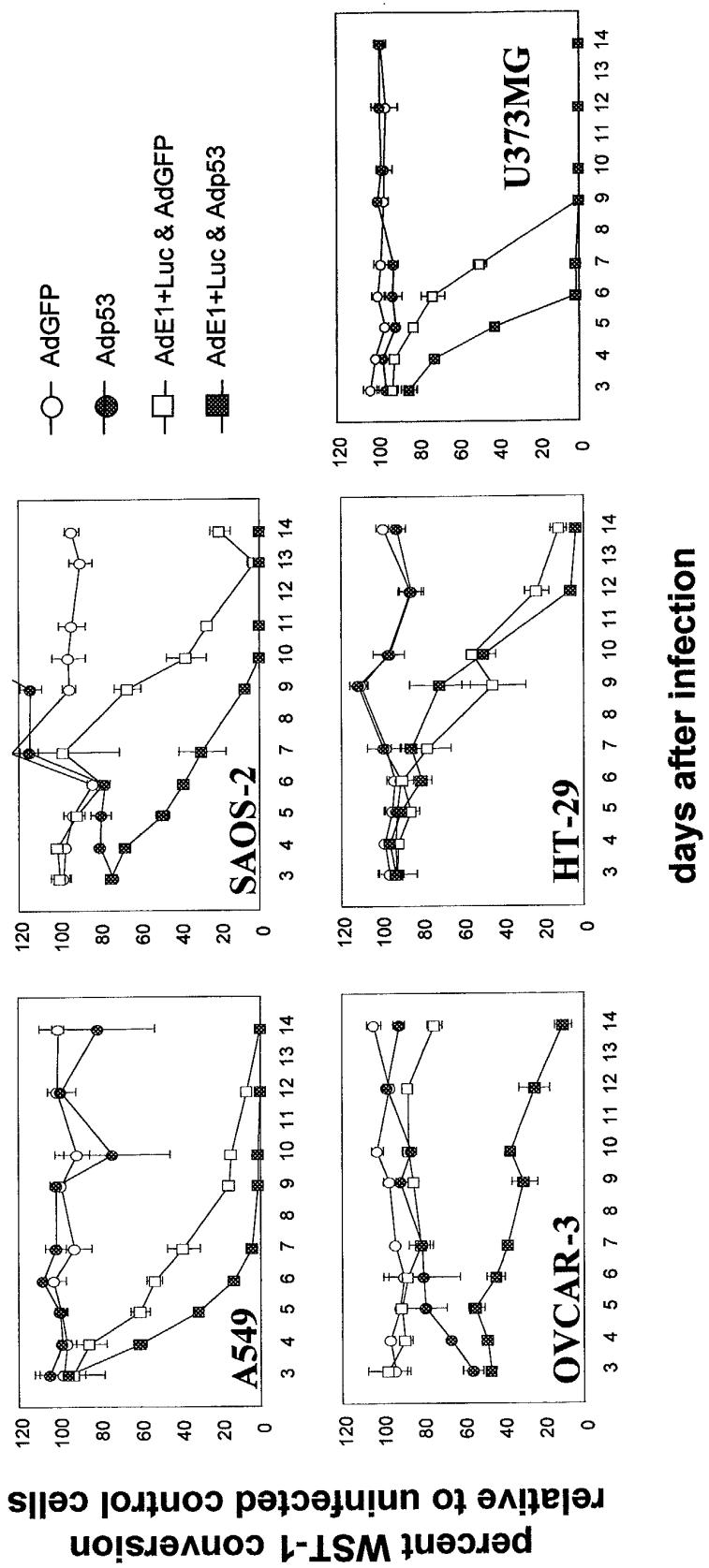
Figure 1

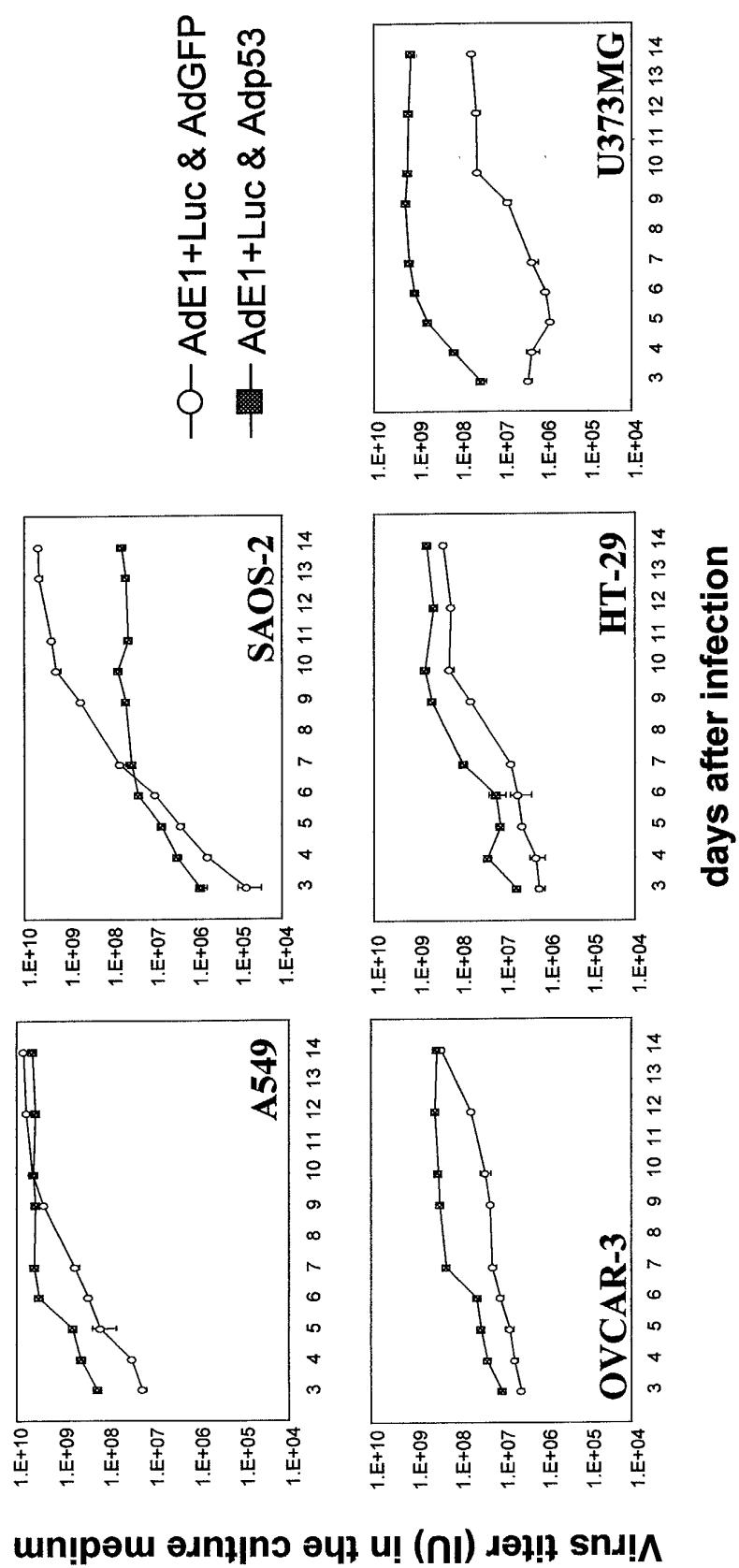
Figure 2

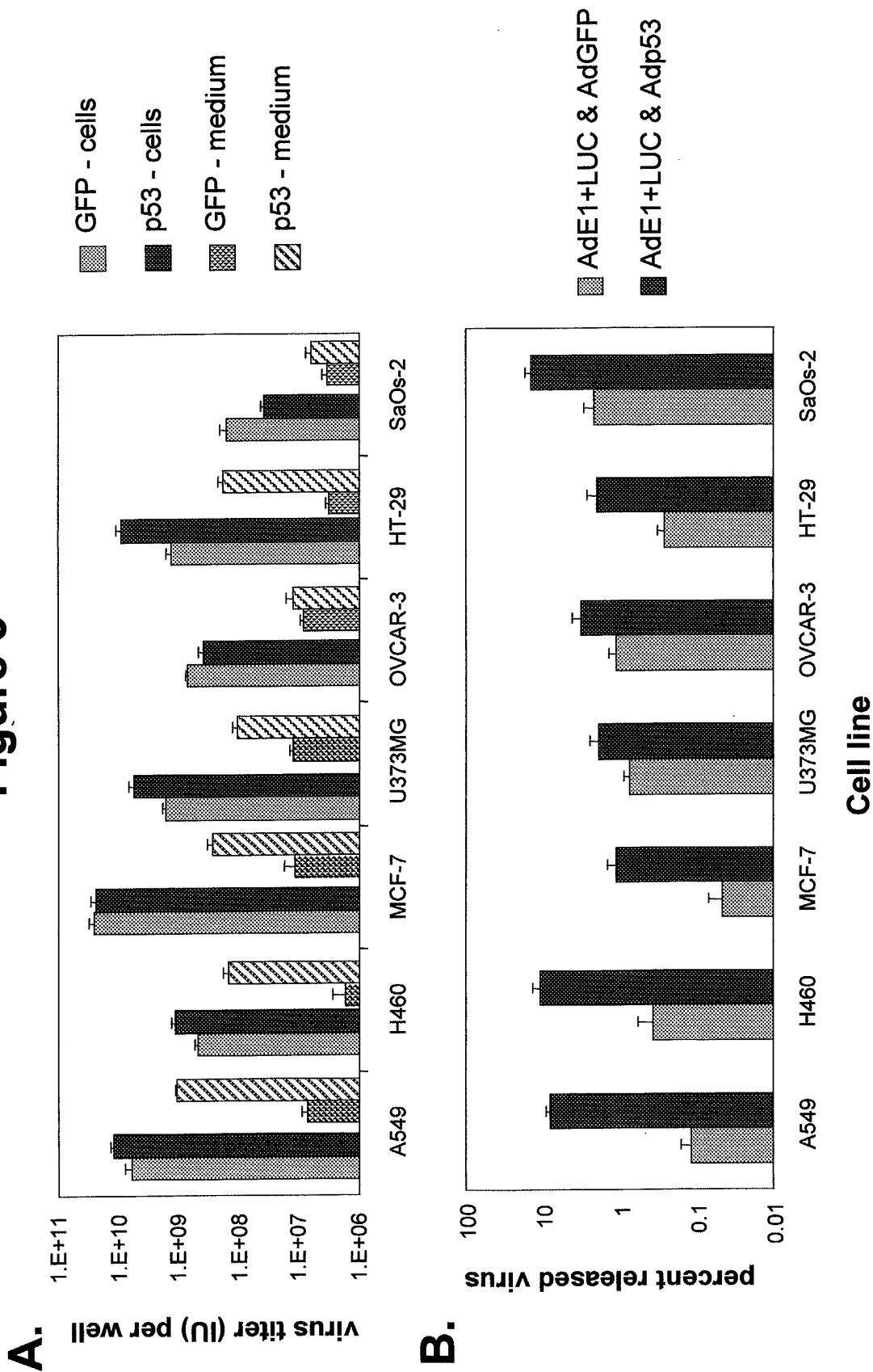
Figure 3

Figure 4

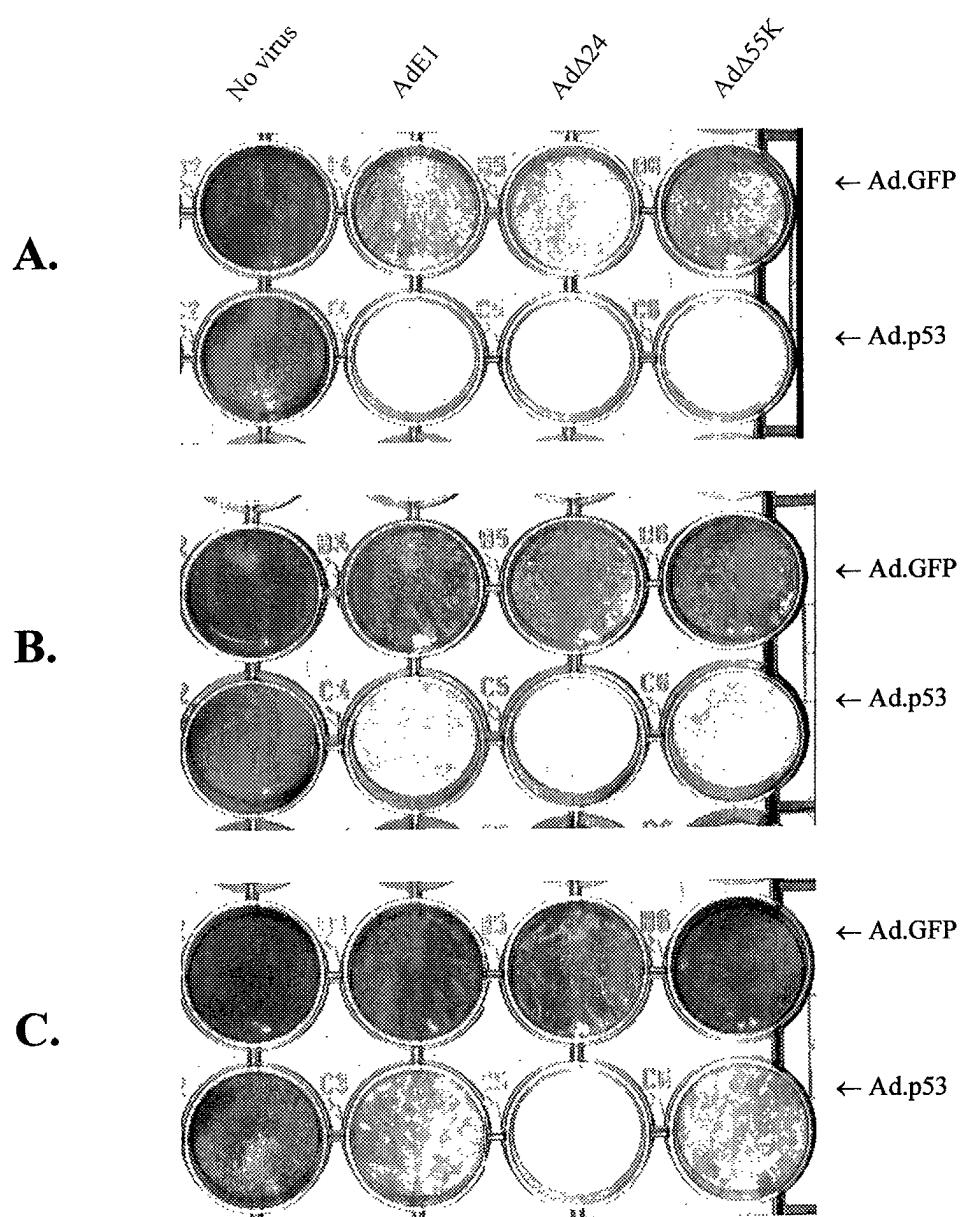


Figure 5 A

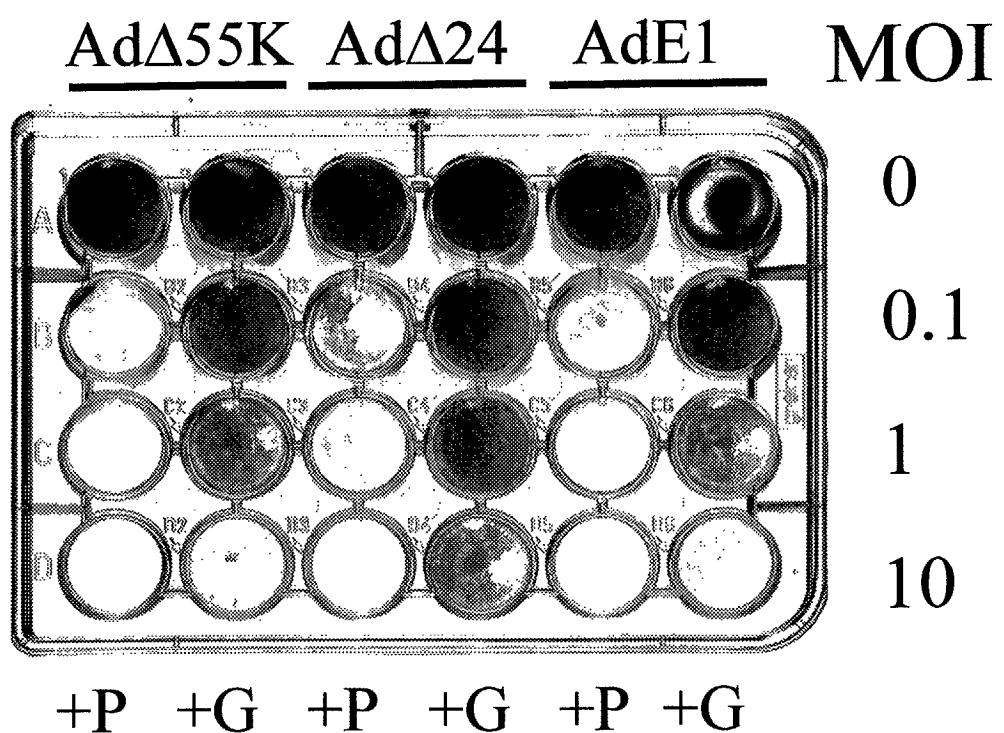
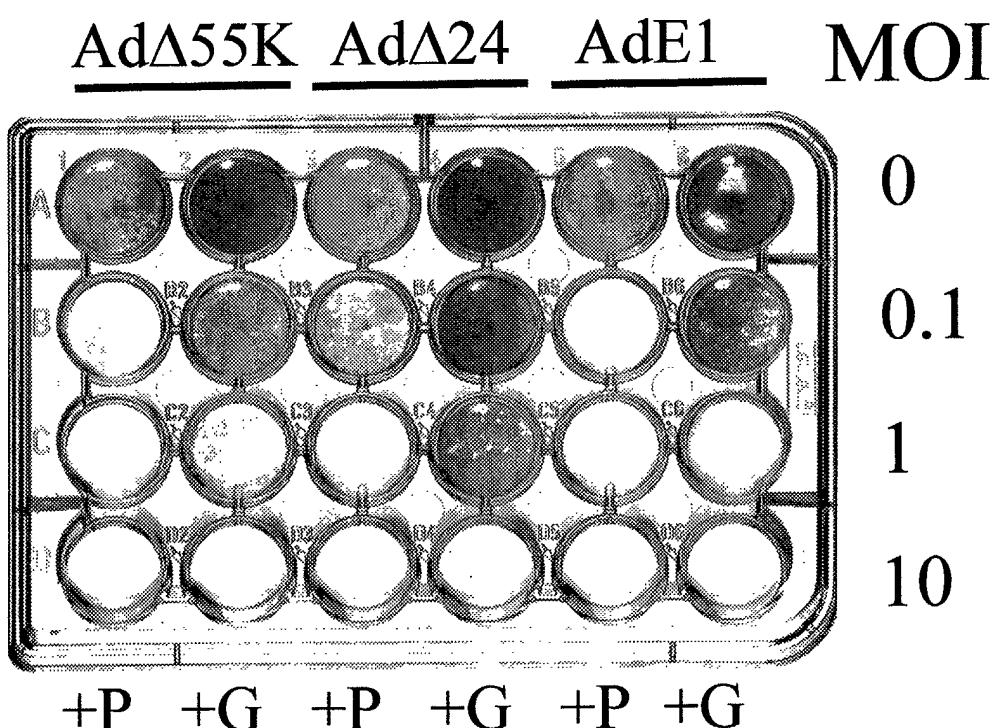
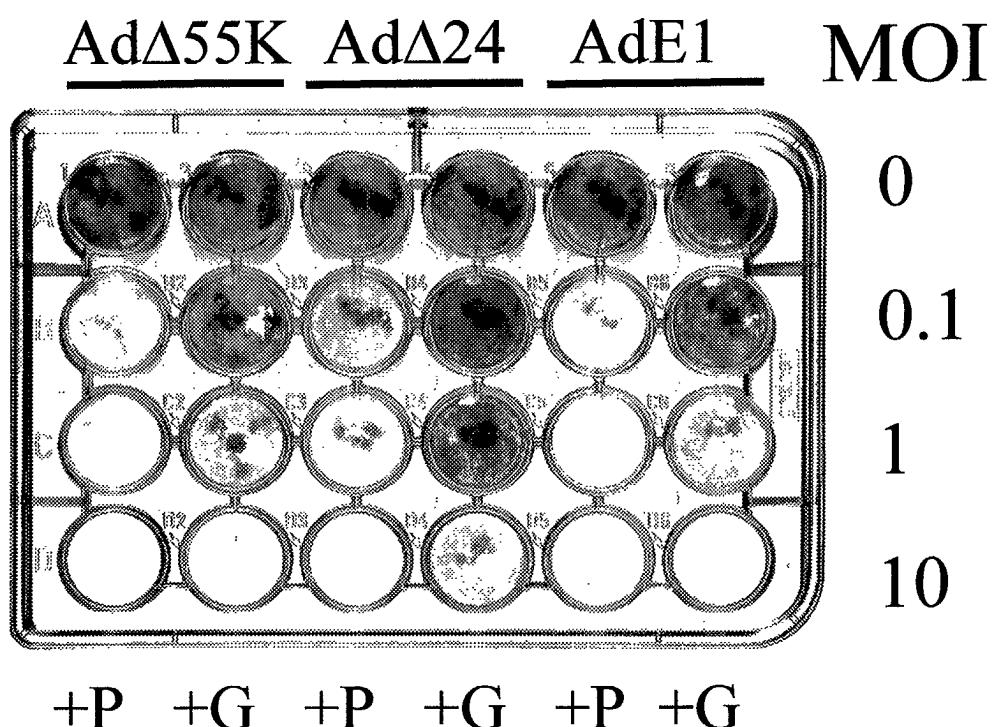


Figure 5 B



SaOs-2

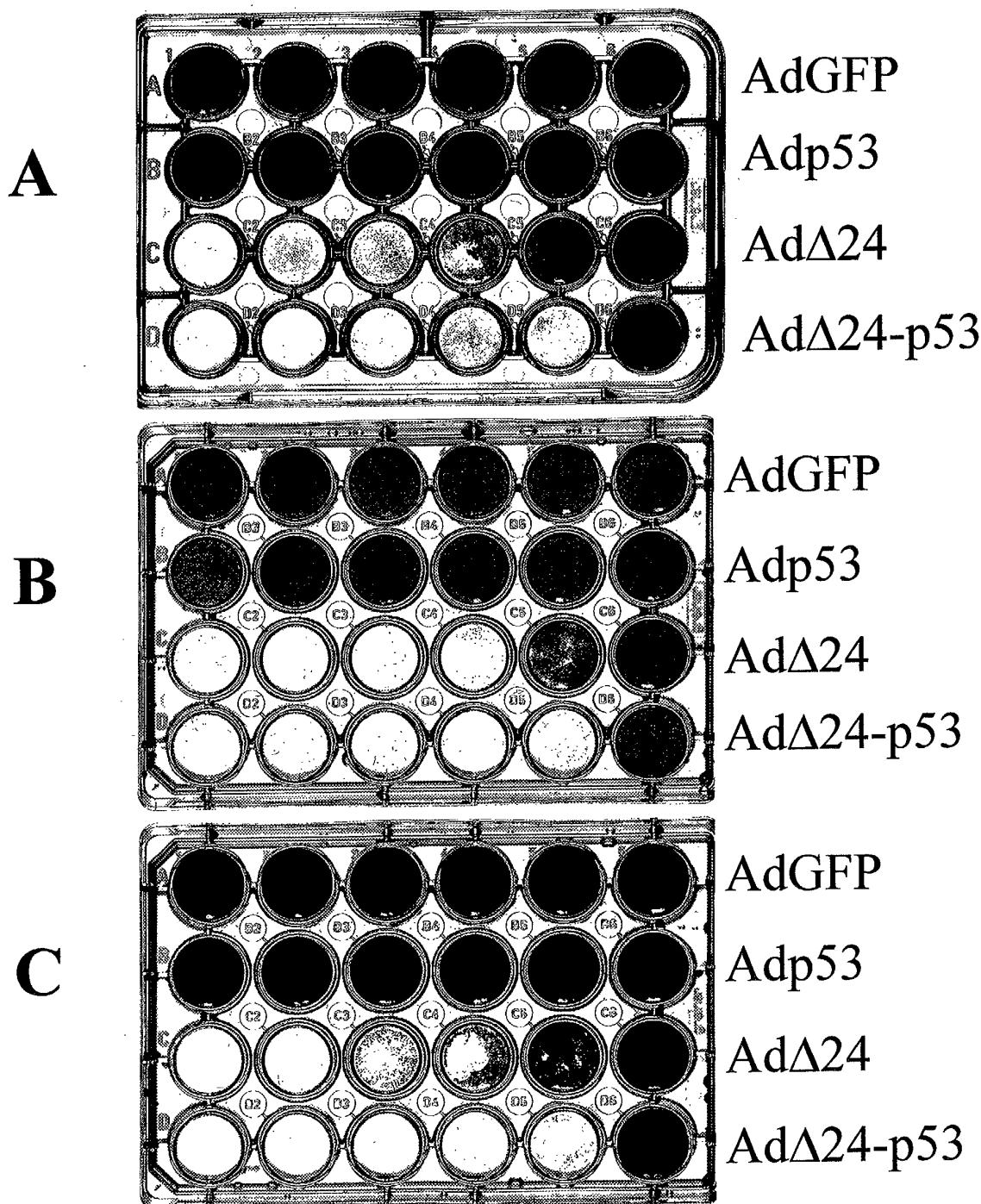
Figure 5 C



U373MG

Figure 6

MOI: 100 10 1 0.1 0.01 0 pfu/cell





DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	WO 98 46779 A (GREGORY RICHARD J ; ARMENTANO DONNA (US); GENZYME CORP (US); KAPLAN) 22 October 1998 (1998-10-22) * abstract * * page 2, line 9 - line 12 * * page 16, line 4 - line 13 * ---	1-11	C12N15/86 C07K14/47
X	WO 97 30732 A (ONYX PHARMA INC) 28 August 1997 (1997-08-28) * the whole document *	1-11	
X	WO 01 74403 A (WOOD CHRISTOPHER BARRY) 11 October 2001 (2001-10-11) * the whole document *	1-4,6-11	
X	WO 01 44280 A (RASTY SIYAMAK ; GENOVO INC (US); HIMES VAUGHN B (US); PELUSO RICHARD) 21 June 2001 (2001-06-21) * abstract * * examples * * claim 22 *	1-4,6-11	
A	---	5	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
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X	WO 00 29573 A (CANJI INC) 25 May 2000 (2000-05-25) * the whole document *	1-4,6-11	

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The present search report has been drawn up for all claims			
Place of search	Date of completion of the search		Examiner
BERLIN	10 June 2002		Panzica, G
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the invention		
Y : particularly relevant if combined with another document of the same category	E : earlier patent document, but published on, or after the filing date		
A : technological background	D : document cited in the application		
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EUROPEAN SEARCH REPORT

Application Number
EP 02 07 5108

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
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X	WO 98 46781 A (GREGORY RICHARD J ;MARSHALL JOHN (US); ARMENTANO DONNA (US); CHENG) 22 October 1998 (1998-10-22) * abstract * * page 8, line 21 - line 29 * ---	1-11	
X	WO 95 12660 A (UNIV TEXAS) 11 May 1995 (1995-05-11) * abstract * * page 7, line 5 - line 23 * * examples * ---	1-4,6-11	
A	WO 98 01563 A (MARCELLUS RICHARD C ;LAVOIE JOSEE N (CA); SHORE GORDON C (CA); TEO) 15 January 1998 (1998-01-15) * figures 1A,1B * ---	1-11	
A	QUERIDO E ET AL: "ACCUMULATION OF P53 INDUCED BY THE ADENOVIRUS E1A PROTEIN REQUIRES REGIONS INVOLVED IN THE STIMULATION OF DNA SYNTHESIS" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 71, no. 5, May 1997 (1997-05), pages 3526-3533, XP002926240 ISSN: 0022-538X * the whole document * ---	1-7	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
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The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
BERLIN	10 June 2002	Panzica, G	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 02 07 5108

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
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<p>The present search report has been drawn up for all claims</p>			
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CATEGORY OF CITED DOCUMENTS			
<p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			



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